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ON THE METAPHOSPHATE OF YEAST. I

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(Received for publication, November 8, 1952)

The presence of metaphosphate in yeast cells was informed by Liebermann (*1*) already in 1888, but the fact left as it was for a long time. In 1944 Mann (*2*) had separated metaphosphate from *Aspergillus niger*, and in 1946 Wiame *et al.* (*3*) reported that, during the fermentation and the respiration of bakers' yeast, inorganic phosphate in media was taken up by yeast cells and then transformed into the metaphosphate.

Metaphosphate might well be regarded as energy rich phosphate as well as important energy source for yeast cells; Lindegren (*4*) informed that metaphosphate played an important role in chromosome division. Wiame's experimental results (*5*) showed that "acid insoluble metaphosphate" was the main source of phosphate of nucleic acid in yeast.

In addition to these informations, there are several other reports on the metaphosphate (*6*), yet we have not any knowledge about the mechanism of the production of metaphosphate, about the manner of its transformation and, above all, about its physiological meanings for yeast cells.

Using the intact yeast cells and the cell-free yeast extract, the authors studied on the production of metaphosphate coupled with their energy metabolism, and particularly on its behaviour in connection with ATP.

EXPERIMENTAL METHODS

Preparation of Phosphate Starved Yeast—According to Wiame *et al.* yeast was treated as follows: Bakers' yeast (Oriental Yeast Co. Ltd.) was washed several times with distilled water, 1–2 g. (wet weight) of which was suspended in 100 ml. of media containing $2\times10^{-3}\,M$ $MgSO_4$, $2\times10^{-2}\,M$ $(NH_4)_2SO_4$, $5\times10^{-3}\,M$ KCl, 1 per cent glucose and $2\times10^{-2}M$ either succinate or glycine buffer at pH 5.0–5.2. The authors adopted succinate buffer solution in the experiment with intact yeast cells, and glycine buffer solution in the experiment with cell-free enzyme solution. This suspension was kept at 30° and violently aerated for a day.

After treated in this way, the yeast cell contained but quite a little quantity of phosphate. It was used for experiment after being washed 4–5 times with distilled water. The pH of media often became acidic after aeration for a day, in spite of the presence of buffer solution; to avoid this trouble, it was recommended to add previously 1 mg. of pantothenate to the media.

Preparation of Cell-free Yeast Extract—The phosphate starved yeast cells (1 g. in wet weight) were nearly dried on an unglazed pottery plate, mixed with glass-wool, ground violently in mortar for 5–10 minutes, suspended in 1 per cent KCl aqueous solution and centrifuged at 3,000–3,500 r.p.m. for about 10 minutes. The supernatant was white turbid and its pH value was about 9.0 owing to the dissolution of glass-well, so it must be adjusted to pH 5.0 with 1/10 N HCl. This solution was used as an enzyme solution.

By the way, this supernatant, if centrifuged at 10,000 r.p.m. for 5 minutes, became almost colorless clear, and did not lose its activity as the enzyme. In the experiments, however, the supernatant solution obtained by centrifuging at 3,000–3,500 r.p.m. was used as an enzyme extract. About 15 ml. of extract were obtained from 1 g. of wet yeast. It was desirable that the whole treatment should be performed at below 10°; the enzyme thus extracted is so unstable even under this condition that it must be prepared freshly just before the experiments.

Fractionation and Estimation of Phosphate Compounds—In general, Wiame's method (*5*) was applied as follows: The " acid-soluble fraction " was extracted from the experimental materials with 5 per cent TCAA (trichloroacetic acid) and the " acid insoluble fraction " from the residue, after being defatted with alcohol-ether, by heating with 5 per cent TCAA. With these fractions, inorganic phosphate, acid labile phosphate (Δ7 min. P) and Ba-insoluble phosphate (metaphosphate fraction) were estimated (metaphosphate of yeast precipitated almost at pH 3.0; the pH on which the precipitated Ba salt was recorded respectively). After heating for 5 minutes with 5 per cent TCAA, about 30 per cent metaphosphate was hydrolyzed, so inorganic phosphate in acid-insoluble fraction may be considered to occur from the acid-labile phosphate. For the estimation of phosphate, the photoelectric colorimetry was applied to the blue color of phosphomolybdate according to Folin's method.

Estimation of Respiration and Fermentation—The oxygen-uptake and the CO_2-output were measured by means of Warburg's manometric apparatus. In case of aerobic respiration, gas space was filled with air, while in case of anaerobic fermentation, with CO_2. The reaction mixture was consisting of $2\times10^{-3}M$ $MgSO_4$, $2\times10^{-2}M$ $(NH_4)_2SO_4$, $5\times10^{-3}M$ KCl, $2\times10^{-3}M$ phosphate, $5\times10^{-2}M$ succinate or glycine buffer at pH 5.0–5.2,* and yeast suspension or yeast extract in main vessel, and 10^{-2} M substrate in side arm (final volume, 3.5 ml.).

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* Succinate was used in the experiment with intact cells and glycine with yeast extract, because succinate was oxidized by yeast extract.

EXPERIMENTAL RESULTS

Experiment with Intact Yeast Cells—By intact yeast cells glucose, galactose, pyruvate, alcohol, α-ketoglutarate *etc.* were oxidized; while citrate, succinate, fumarate, tartarate, glutarate *etc.* were not oxidized (Fig. 1). During the respiration, inorganic phosphate in media gradually decreased. The absorption of phosphate occurred during the oxidation of glucose, pyruvate, alcohol *etc.* (Fig. 2). While the phosphate-uptake did not take place with substrates such as citrate, succinate *etc.* which were not oxidized by intact yeast cells. With respect to acetate,

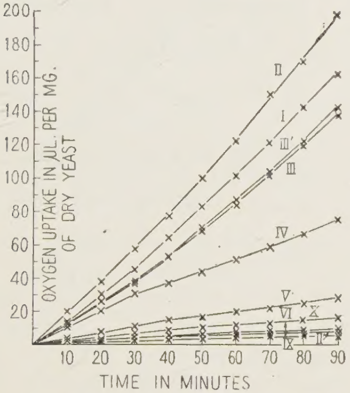

FIG. 1. O_2-uptake by intact yeast cells.

The reaction mixture contained $2\times10^{-2}M$ $MgSO_4$, $2\times10^{-2}M$ $(NH_4)_2SO_4$, $5\times10^{-3}M$ KCl, $2\times10^{-3}M$ phosphate, $5\times10^{-2}M$ succinate buffer at pH 5.0–5.2, $10^{-2}M$ substrate and intact yeast cells; total volume 3.5 ml.; 0.3 ml. of 10 per cent KOH in center well. Air in gas space; temperature at 37°.

I Glucose. II Alcohol. II′ Alcohol+$5\times10^{-5}M$ 2,4-DNP. III Acetate. III′ Acetate +5 mg. pantothenate. IV Pyruvate. V α-Ketoglutarate. VI Fumarate. VII Glutarate. VIII Citrate. IX Tartarate. X No substrate.

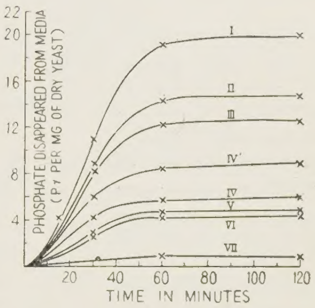

FIG. 2. Phosphate disapearance from media

The reaction mixture was same as in the case of Fig. 1. In the Experiments VI and VII, orthophosphate was replaced by metaphosphate and pyrophosphate, respectively.

I Orthophosphate+glucose. II Orthophosphate + pyruvate. III Orthoposphate+alcohol. IV Orthophosphate+acetate. IV′ Orthophosphate + acetate + 5 mg. pan tothenate. V Orthophosphate+ citrate or fumarate or tartarate or glutarate or no substrate. VI Metaphosphate + glucose, VII Pyrophosphate+glucose.

oxidation took place, but the amout of phosphate-uptake was very small without the addition of pantothenate. The phosphate-uptake in this way was estimated in several fractions (Table I). Almost all of the phosphate taken up was recovered in the form of acid-insoluble acid-labile fraction, greater part of which was thought to be metaphosphate. This fraction was precipitated as Ba-salt at pH 4.0, washed twice with water, dissolved in an adequate quantity of 0.01 N H_2SO_4, and, after centrifugation, the supernatant was neutralized to pH 7.0. When a few drops of toluidin-blue solution was added to it, it caused distinct metachromatic reaction. When gulcose was used as substrate, the influences of several reagents upon O_2-uptake and phosphate-uptake of yeast cells were shown in Fig. 3 and Fig. 4, respectively. 2,4-Dinitrophenol (DNP) at a concentration of 5×10^{-5} M, a representative inhibitor of

TABLE I

Estimation of Several Sorts of Phosphate in Yeast Cells

Condition of yeast	Phosphate disappeared from media (per mg. dry yeast)	Phosphate in yeast cells (per mg. dry yeast)		
		Acid-soluble		Acid-insoluble
		Inorg. P	Δ7 min. P	Inorg. P+Δ7 min. P
	γ P	γ P	γ	γ
Phosphate starved yeast	0	<0.1	0.23	1.47
Aerated with glucose and phosphate for 30 minutes....................	11.2	2.05	1.42	7.32
Aerated with glucose and phosphate for 90 minutes....................	20.1	1.47	0.83	16.9
Aerated with phosphate and no substrate for 90 minutes....................	4.8	1.0	0.4	2.8
Incubated with glucose and phosphate under anaerobic condition for 60 minutes	17.5	1.6	2.1	12.5

The reaction mixture contained $2 \times 10^{-3} M$ $MgCl_2$, $5 \times 10^{-3} M$ KCl, $2 \times 10^{-3} M$ phosphate, $5 \times 10^{-2} M$ succinate buffer at pH 5.0–5.2, $10^{-2} M$ glucose and phosphate starved yeast cells. The yeast cells were washed twice with distilled water by centrifugation, and extracted with 5 per cent trichloracetic acid (acid-soluble fraction). The acid-insoluble fraction was extracted from the residue, after being defatted with alcohol-ether, by heating with 5 per cent trichloracetic acid.

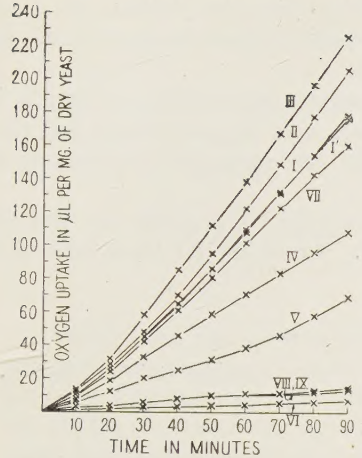

Fig. 3. Influences of the several reagents upon O_2-uptake.

The reaction mixture contained $2 \times 10^{-3} M$ $MgSO_4$, $2 \times 10^{-2} M$ $(NH_4)_2SO_4$, $5 \times 10^{-3} M$ KCl, $2 \times 10^{-3} M$ phosphate, $5 \times 10^{-2} M$ succinate buffer at pH 5.0–5.2, $10^{-2} M$ substrate, yeast cells and reagent indicated; total volume 3.5 ml.; 0.3 ml. of 10 per cent KOH in center well. Air in gas space; temperature at 37°

I Glucose. I′ Glucose + $10^{-3} M$ $BeSO_4$. II Glucose + $10^{-5} M$ 2,4-DNP. III Glucose + $5 \times 10^{-5} M$ 2,4-DNP. IV Glucose + $10^{-4} M$ 2,4-DNP. V Glucose + $10^{-3} M$ arsenate. VI Glucose + $10^{-2} M$ arsenate. VII Glucose + $10^{-2} M$ NaF. VIII No substrate + $5 \times 10^{-5} M$ 2,4-DNP. IX No substrate.

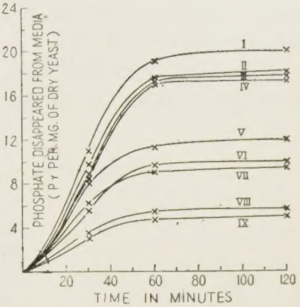

Fig. 4. Influence of the several reagents upon phosphate disappearance.

The reaction mixture was the same as in the case of Fig. 3. In the Experiments II, CO_2 in gas space and no KOH in center well.

I Glucose. II Glucose (anaerobic). III Glucose + $10^{-5} M$ 2,4-DNP. IV Glucose + $10^{-3} M$ $BeSO_4$ + $10^{-1} M$ $MgSO_4$. V Glucose + $10^{-2} M$ NaF. VI Glucose + $10^{-4} M$ arsenate. VII Glucose + $10^{-3} M$ $BeSO_4$. VIII Glucose + 5 $10^{-5} M$ 2,4-DPN. IX No substrate

the oxidative phosphorylation, raised the respiration, but completely stopped the phosphate-uptake. As to Be^{++}, it was reported lately that it was poisonous to animal and alkaline phosphatase (*7*). According to Du Bois (*8*), Be^{++} inhibited ATPase, but opposite results were also presented by Klemperer (*9*). $10^{-3} M$ Be^{++} did not decreace the O_2-uptake, but strongly inhibited the phosphate-uptake. This inhibition was abolished by $10^{-1} M$ Mg^{++}. It is yet unknown whether Be^{++} has any inhibitory effect upon the oxidative phosphorylation by "cyclophorase system" of animals. NaF and arsenate stopped both respiration and phospahte-uptake.

When metaphosphate or pyrophosphate was added to media in place of orthophosphate, metaphosphate was slightly taken up, but pyrophosphate not at all. These results are agreeable with Wiame's experimental results (3).

Experiment with Cell-Free Yeast Extract—The O_2-uptake by the extracted yeast juice is shown in Fig. 5. Considerable O_2-uptake took place even without substrate. After 40 minutes or so, the O_2-uptake nearly ceased, which showed that the extracted juice did not contain any living yeast cells.

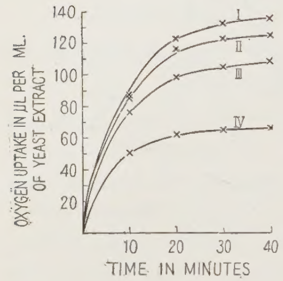

FIG. 5. O_2-uptake by the yeast extract.

The reaction mixture contained $2\times10^{-3}M$ $MgSO_4$, $2\times10^{-2}M$ $(NH_4)_2SO_4$, $5\times10^{-3}M$ KCl, $2\times10^{-3}M$ phosphate, $5\times10^{-2}M$ glycine buffer at pH 5.0, 10^{-2} M substrate and 1 ml. of yeast extract; total volume 3.5 ml.; 0.3 ml. of 10 per cent KOH in center well. Air in gas space; temperature at 25°.

I Succinate. II Alcohol. III Glucose. IV No substrate

This enzyme solution absorbed O_2, when glucose, alcohol, pyruvate, α-ketoglutalate, acetate, citrate, succinate or fumarate was added as substrate, and during this process the phosphate in media disappeared (Table II). The values in the table are those which the none-substrate O_2-uptake is subtracted. Notwithstanding the similar extracts prepared under the same conditions were used, the values of the O_2-uptake and the amounts of phosphate disappeared varied from experiment to experiment. Without substrate, it showed almost always no phosphate-uptake. Succinate, citrate *etc.*, which were not oxidized by intact yeast cells, were used by this extract.

DNP and Be^{++} inhibited the phosphorylation by the extract as in case of intact yeast cells. The inhibitory action of Be^{++} was weakened by the addition of Mg^{++}. While intact yeast cells need K^+ for the absorption of phosphosphate (Schmidt *et al.* (*3*)), the extracted solution without K^+ also did not take up phosphate.* So it is clear that the effect of K^+

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* In this experiment, the media contained no KCl; in preparation of the yeast extract, NaCl was adopted in place of KCl.

TABLE II

Oxygen-uptake and Phosphate-uptake by the Yeast Extract

Exptl. No.	Substrate added	O_2-uptake† in 40 minutes	Phosphate-uptake† in 40 minutes
		$\mu l.$	γP
1	Alcohol	54.9	24±2
	Pruvate	73.1	21 "
	α-Ketoglutarate	59.1	21 "
	Acetate	39.7	15 "
	Fumarate	38.9	18 "
2	Alcohol	52.4	66 "
	Glucose	28.1	54 "
3	Acetate	26.6	18 "
	Citrate	23.1	27 "
4	Glucose	28.0	28 "
	Glucose (anaerobic)	84.0††	25 "

The reaction mixture contained $2\times10^{-3}M$ $MgSO_4$, $2\times10^{-2}M$ $(NH_4)_2SO_4$, $5\times10^{-3}M$ KCl, $2\times10^{-3}M$ phosphate, $5\times10^{-2}M$ glycine buffer at pH 5.0, $10^{-2}M$ substrate and 1 ml. of yeast extract; total volume 3.5 ml.; 0.3 ml. of 10 per cent KOH in center well. Air in gas space; temperature at 25°.

† No substrate uptake was subtracted from these values.

†† CO_2 produced. In this case CO_2 in gas space, and no KOH in center well.

is merely to raise the degree of permeability of cell membrane toward phosphate.

Nicotinic acid amide, ATP, creatine *etc.* gave no influence upon respiration as well as phosphate-uptake. Even under anaerobic condition, phosphate-uptake took place with glucose as a substrate (Table III).

The of O_2-uptake by this yeast extract was found to be optimal at pH 5.5-3.5, and it suddenly decreased in acid side of this pH, and alkaline side as well.

It is very important to investigate the compounds, to which this absorbed inorganic phosphate is transformed. For this purpose, a 10 ml.-portion of the reaction mixture (using alcohol as a substrate) was aerated

TABLE III

Influences of the Several Reagents upon Oxygen-uptake and Phosphate-uptake by Yeast Extract

Exptl. No.	Substrate added	Reagent added	O^2-uptake† in 40 minutes	Phosphate-uptake† in 40 minutes
			$\mu l.$	γP
1	Glucose	None	37.6	48±2
	Glucose	$10^{-4}M$ 2,4-DNP	0	0 ”
	Glucose	$3\times10^{-5}M$ 2,4-DNP	35.9	15 ”
2	Alcohol	None	49.9	28 ”
	Alcohol	$10^{-3}M$ $BeSO_4$	52.7	0 ”
	Alcohol	$10^{-3}M$ $BeSO_4$ $10^{-1}M$ $MgSO_4$	45.2	21 ”
3	Alcohol	None	60.7	28 ”
	Alcohol	KCl omitted	41.7	0 ”
	Alcohol	$MgSO_4$ omitted	50.4	21 ”
4	Alcohol		43.3	34 ”
	Alcohol	$10^{-2}M$ NaF	37.3	0 ”
	Alcohol	$10^{-3}M$ Arsenate	31.3	20 ”
5	Alcohol		33.5	39 ”
	Alcohol	$10^{-2}M$ Nicotinic acid amide	28.6	39 ”
	Alcohol	$3\times10^{-3}M$ ATP	31.5	39 ”
	Alcohol	$10^{-2}M$ Creatine	30.2	39 ”

The reaction mixture was the same as in case of Table II.

† No substrate uptake was subtracted from these values.

for 1 hour at 30° and then protein of the reaction mixture was precipitated with 5 per cent TCAA and centrifuged off. With this supernatant solution, the estimation of orthophosphate, acid-labile phosphate and phosphate precipitated with barium acetate at pH 4.0-3.5 were carried out (Table IV). It was recognized that the almost all of the phosphate disappeared was present in metaphosphate fraction. This Ba-salt showed metachromatic reaction to toluidin blue (the treatment was the same as in case of intact yeast cells). This fraction was decomposed by metaphosphatase prepared from rice bran (*10*). So it

TABLE IV

Phosphate Disappearance from Media and Metaphosphate Formation by the Yeast Extract

Reaction mixture	Phosphate disappeared from media	Δ7 min. P produced	Ba-insoluble acid-labile phosphate P (metaphosphate fraction)	Metacromatic reaction
	γP	γ	γ	
Complete	204	226†	167 (precipitate at pH 4.0)	+
Complete	62		57 (precipitate at pH 3.5)	+
Replaced phosphate with $5\times10^{-3}M$ ATP			††	+
Replaced phosphate with $5\times10^{-3}M$ ATP and omitted alcohol			††	+

The complete reaction mixture contained $2\times10^{-3}M$ $MgCl_2$, $5\times10^{-3}M$ KCl, $2\times10^{-3}M$ phosphate, $5\times10^{-2}M$ glycine buffer at pH 5.0, $10^{-2}M$ alcohol and yeast extract. After aeration for 1 hour at 30°, protein of the reaction mixture was precipitated with 5 per cent trichloracetic acid and centrifuged off. With this supernatant solution, the estimation of orthophosphate, acid-labile phosphate and phosphate precipitated with barium acetate were carried out.

† Acid-insoluble acid-labile phosphate P was 10 γ.

†† ATP used (about 90 per cent purity from acid-labile phosphate) was partly precipitated with barium acetate at pH 3.5–4.0. So the quantitative estimation of metaphosphate fraction could not be carried out.

is apparent that the phosphate absorbed is almost transformed into higher metaphosphate.*

Metaphosphate formation also took place with ATP in place of inorganic phosphate. Furthermore, when ATP was used, metaphosphate was also formed without substrate (Table IV).

SUMMARY

It was found that, by intact yeast cells as well as extracted juice, inorganic phosphate in media was transformed into higher metaphosphate, accompanied by the oxidation of glucose, alcohol *etc.* or with

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* In intact yeast cells, metaphosphate was rich in acid-insoluble fraction; while in the yeast extract acid-insoluble fraction, as shown in Table IV, it was so poor. The distinction between acid-soluble and -insoluble fraction is only convenience, and theae is no difference in chemical nature between the two phosphate compounds (*6*).

fermentation. Inorganic phosphate may be transformed partly to inorganic pyrophosphate in the process of oxidative phosphorylation caused by the " cyclophorase system " of animal organs (*11*). The reversible enzymatic synthesis of pyrophosphate from ATP was found by Kornberg *et al.*; but in Kornberg's reactions pyrophosphate could not be replaced by metaphosphate (*12*). In our experiments, it has been shown that metaphosphate may be synthesized from ATP by yeast extract; but it has not yet been clarified whether this reaction is reversible or not.

In conclusion the authors should like to thank Prof. T. Soda for his valuable guidance and encouragement throughout this study.

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BIOCHEMICAL STUDIES ON THE NUMBER AND THE COMPOSTION OF LIVER CELLS IN POSTNATAL GROWTH OF THE RAT

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(Received for publication, November 10, 1952)

In the last few years the number and the composition of cells in growing tissues have been of common interest among cytochemists and cell physiologists (*1–4*). The growth of the liver was investigated by Campbell and Kosterlitz (*5*), but their results did not cover the period of the most active growth. The study of this type was performed in this laboratory with livers of rats in postnatal growth. It contains estimation of the number of nuclei and determination of DNA, PNA, and protein contents per liver as well as per average liver cell. Here, the number of nuclei is regarded as a functional representative of the cell number.

In some of similar studies already published with different materials (*1*, *2*, *3*), cell number of a given specimen was estimated from the quantities of DNA per specimen and per average nucleus, the latter being estimated with nuclei isolated from separate materials. This method is based upon the assumption that the average DNA content of the cell is constant in all the specimens under investigation. With livers, however, the situation is not so simple because of the presence of polyploid nuclei, which are more abundant in mature animals than in younger ones (*6*, *7*). Accordingly, DNA content of average liver nuclei must be estimated with each of the specimens studied. With regenerating livers, Price and Laird (*4*) attempted to avoid such a difficulty by counting nuclei directly with fresh homogenates. But with this method we could not obtain any reliable results. We employed here the method of Cunningham, Griffin and Luck (*8*) with some modification for determing the DNA content of average nuclei. Results obtained were published in part in a preliminary report (*9*).

MATERIALS AND METHODS

Animals—Infant, young and adult rats were obtained from a colony maintained in this laboratory through repeated inbreedings. The body weight of animals was taken as indicative of the growth stage. Beginning with baby rats in which the hepatic hematopoiesis is disappearing almost completely, we examined six growth stages with body weights of 12, 25, 50, 100, 200, and 340 g. The fluctuation of the body weight did not exceed 5 per cent. The first two classes consisted of sucklings of mixed sexes; with animals of 50 to 200 g. body weights, both sexes were treated separately; and the last one class comprised males only. With each of these groups three independent determinations were made, each time with a single rat, except for 12 and 25 g. rats, with which livers of 5 and 2 animals were pooled for one determination, respectively.

Male adult rats weighing 200 g. were subjected to prolonged starvation for 7–11 days until some of them became moribund. Of these, three seemingly dying rats were subjected to the examination of the liver. Young rats of mixed sexes, weighing about 38 g. were fed on stock diet restricted in quantity, so as to keep the body weight of the animals below 50 g. for 4 weeks. During the same length of time, the body weight of animals fed on sufficient quantity of the stock diet increased up to 130–150 g.

Procedure—Livers were removed quickly after bleeding by decapitation and weighed. Livers of rats in starvation and malnutrition were then perfused with ice-cold saline. In normally growing rats, the perfusion was omitted, since no essential difference of the data was experienced between perfused and non-perfused portions of the same liver.

After removing a small piece for histological observations, the remaining bulk of the liver was reweighed for correction of this loss, and homogenized in a Potter-Elvehjem glass homogenizer with ice-cold 2 per cet citric acid to give about 10 per cent homogenate (I). Aliquots of I (2 ml.) were removed for determination of nucleic acids and protein contents of the whole liver. The remainder of the homogenate was diluted 4 to 8-fold with ice-cold 2 per cent citric acid, strained through 8 layers of gauze and centrifuged for 2.5 minutes at $36 \times g$ (500 r.p.m.) to eliminate undisintegrated tissue masses and cell debris. Only a small proportion of the liberated nuclei was sedimented. The supernate (II) was a completely homogeneous suspension of nuclei and cytoplasmic materials. This procedure made the nuclear count exceedingly precise*. Then, the following procedure was conducted in duplicate: A known volume (20 ml.) of II was centrifuged for 8 minutes at $36 \times g$ to throw down more than 50 per cent of the nuclei.

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* Counting nuclei with suspensions derived directly from orginal homogenates, as conducted by Price *et al.* (*4*, *10*) proved to be inaccurate with homogenates prepared in Potter-Elvehjem homogenizer, because remaining small tissue masses decidedly caused error of significant magnitude. On the other hand, homogenization in Waring blendor was found to break a considerable proportion of the nuclei; results obtained from Waring blendor homogenates might, therefore, involve a serious error, whether nuclear count is performed with original homogenate or after vigorous purification of ioslated nuclei.

The sediment was resuspended in ice-cold 2 per cent citric acid solution and centrifuged again, and the final sediment (III) was subjected to DNA determination. The supernates were combined (IV). The number of nuclei in III was obtained by difference from nuclear counts of II (average of two diluted portions) and of IV after appropriate dilution with citric acid. Nuclei were counted with Bürker hemocytometer twice in five squares (0.1 mm.^{3}) for each diluted portion. Differential counting of parenchymal cell nuclei and nuclei of other cell types, for which the presence of prominent nucleoli in the former served as a good criterion, proved that there was practically no tendency of preferential sedimentation of the larger parenchymal nuclei in the low-speed centrifugation employed.

Since the partial sedimentation of nuclei which separates III and IV did not proceed quite uniformly in duplicate tubes run simultaneously, average DNA content of the sedimented nuclei in the duplicate tubes were computed separately from respective figures on DNA content and number of nuclei, the final value of DNA per nucleus being the mean of these two determinations. DNA-P, PNA-P, and protein N (P-N) contents of the original homogenate (I), being determined also in duplicate, were converted into the quantities per liver with corrections made by weight ratio for the loss of a small piece removed for morphological observation. Then, number of nuclei in the whole liver was calculated from DNA contents per liver and per nuclei. Quantities of PNA and P-N per cell were obtained conversely from values per liver and the number of nuclei. Mean cell mass was given by dividing liver weight with number of nuclei per liver. Quantities of nucleic acids per liver refer to P contents, but those per cell are expressed in mass of the whole substance on the basis of tetranucleotide formulae.

Analytical Method—Nucleic acids were determined by Schneider method (*11*); diphenylamine and orcinol tests served for measurements of DNA-P and PNA-P, respectively (*12*). Standard sample of DNA used was the same as indicated elsewhere (*13*). That of PNA was yeast Na ribonucleate prepared by Clarke-Schryver method (*14*) (P content: 9.0 per cent)*. Protein N (P-N) was determined with the hot trichloracetic acid residue using digestion with H_2SO_4-H_2O_2 followed by hypobromite-iodometry (*12*).

Estimation of Error of Methods—Few papers so far published on chemical determinations in cell unit contained statistical evaluations of errors involved and data obtained. Here, we will follow the method of Goldberg, Klein and Klein (*15*), mainly for the convenience of comparing our data with theirs as regards the magnitude of errors. Experimental errors involved in nuclear counts and biochemical determinations are estimated from differences of duplicates of all the samples examined in this study, and summarized in Table I. For calculating per cent standard deviation, or variation coefficient, all the figures are first converted into per cent of mean of respective classes. Variability among animals is calculated from standard deviation and error of methods (Table II).

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* Kindly supplied by Dr. M. Shimomura, Department of Chemistry, University of Nagoya.

TABLE I

*Error of Methods Established from Differences of Duplicates**

Method**	Number of duplicates***	Mean of all samples	Standard deviation (σ_x) of single determination	Experimental error of method (ε_m): mean of duplicates	
				ε_m	Per cent of mean
1 Nucleus 10^6 per ml. of II	44	65.8	0.99	0.70	1.1
2 DNA μg. P per ml. of I	41	30.5	0.44	0.31	1.0
3 PNA μg. P per ml. of I	36	102.7	1.27	0.90	0.9
4 Protein mg. N per ml. of I	36	2.84	0.10	0.074	2.6
5 DNA μμg. per nucleus	38	9.81	0.18	0.13	1.3 (1.6****)

* Standard deviation of single determination (σ_x) is cacld. by the formula: $\sigma_x = \sigma_d/\sqrt{2}$, where σ_d is the stand. dev. of the diff. between duplicates. As each of the final values obtained with a single specimen is the mean of duplicate determinations, the error of the method (ε_m) is given by $\varepsilon_m = \sigma_d/\sqrt{2} = \sigma_x/\sqrt{2}$. Usually this value is obtained by averaging several classes divided according to the magnitude of mean.

** For the Roman numerals indicated, see p. 96–97 (Procedure).

*** Some additional determinations are included.

**** Calculated from errors of nuclear count and DNA detmn.: Error of nuclear count in III (ε_{mIII}) $= \sqrt{\varepsilon_{mII}^2 + \varepsilon_{mIV}^2}$, because the number of nuclei in III (n_{III}) is obtained from n_{II} and n_{IV} by difference. $\varepsilon_{mII} = 1.1$ (line 1), while ε_{mIV} can not be calcd., because IV was obtained in single unit for each III; ε_{mIV} is therefore replaced with σ_x of the nuclear count of II, viz., $1.1 \times \sqrt{2} = 1.5$. Thus, $\varepsilon_{mIII} = \sqrt{1.1^2 + 1.5^2} = 1.8$ per cent. Then, σ_x of the detmn. of DNA per nucleus in a single unit $= \sqrt{1.8^2 + 1.4^2} = 2.3$ per cent, where 1.4 is σ_x (in per cent of mean) of DNA detmn. in a single unit of III (substituted by per cent σ_x of DNA detmn. in I (line 2)). As the final value of DNA per nucleus is obtained as a mean of duplicate detmns., ε_m of determining DNA per nucleus $= 2.3/\sqrt{2} = 1.6$ per cent, a value not far from that calculated directly with experimental values of the duplicates.

TABLE II

Experimental Error of Methods, Standard Deviation, and Variability among Animals of Various Determinations, as Expressed in Per cent of Mean

	Factor	Experimental error of method	Standard deviation of data	Variability among animals
1	Number of cells per liver	1.6*	9.4	9.3
2	DNA-P per liver	1.0**	9.4	9.3
3	PNA-P per liver	0.9**	5.7	5.6
4	P-N per liver	2.6**	10.9	10.6
5	DNA per cell	1.3	2.4	2.0
6	PNA per cell	1.9***	9.3	9.1
7	P-N per cell	3.1***	13.3	12.9

Column 2: For number of specimens, see Table I.

Column 3: Average of standard deviations calcd. from figures expressed in per cent of means of respective groups. Number of specimens=27 for all the factors.

Column 4: Calculated from columns 2 and 3$=\sqrt{\text{col. } 3^2-\text{col. } 2^2}$

* Calulated from errors of DNA per liver (line 2) and DNA per cell (line 5)$=\sqrt{\text{line } 2^2+\text{line } 5^2}$.

** These are assumed to be equal to errors in determination of the quantities per ml. of the original homogenate (I) (Table I) which was prepared in single unit. The actual errors should be somewhat higher, because errors involved in correcting for the loss of small fractions removed for the morphological observation were neglected, the correction for this factor being impossible in the present case. However, the error due to this factor must be far less than 5 per cent, because the removed pieces always did not exceed 5 per cent of the whole liver in weight.

*** Calculated from errors of cell number (line 1) and of PNA or protein per liver (line 3 or 4, resp.)$=\sqrt{\text{line } 1^2+\text{line } 3(4)^2}$.

RESULTS

Results obtained with rats in postnatal growth are summarized in Table III. These are largely based on the same materials as employed in a previous report (*9*).

In Table III, the growth of the liver is expressed in terms of liver weight, cell number, and average cell mass. The cell number represents the whole nuclear population of the liver, regardless of cell types. From differential counting of liver nuclei, the proportion of parenchymal

TABLE III

Nucleic Acids, Protein and Cell Number of Rat Liver Tissues in Postnatal Growth

(Average of three determinations; number of animals in a single determinations is 5, 2, 1 for 12 g., 25 g., and 50 through 340 g., respectively.)

Body weight	Sex	Age	Liver weight	Cell number	Quantity per live			$\frac{\text{PNA-P}}{\text{DNA-P}}$	$\frac{\text{PNA-P}}{\text{P-N}}$	Quantity per 100 g liver			Quantity per average cell			Cell mass
					DNA-P	PNA-P	P-N			DNA-P	PNA-P	P-N	DNA	PNA	P-N	
g.		*Days*	*g.*	$\times 10^{-6}$	*mg.*	*mg.*	*mg.*			*mg.*	*mg.*	*g.*	*μμg.*	*μμg.*	*μμg.*	*mμg.*
12	—	10	0.30	168	0.098	0.17	4.95	1.76	0.036	33.3	58.4	1.67	5.90	10.4	29.3	1.79
25	—	21	0.98	445	0.223	0.50	19.4	2.29	0.027	23.6	52.2	1.96	5.09	11.6	45.6	2.20
50	♂	31	2.6	668	0.614	2.34	52.1	3.62	0.042	23.6	85.4	2.01	9.27*	33.5	78.0	3.89
50	♀	32	2.7	692	0.626	2.34	56.8	3.74	0.042	22.9	85.7	2.07	9.14	34.2	81.9	3.90
100	♂	41	5.7	1060	1.17	4.84	112	4.21	0.043	20.7	85.3	1.98	11.1	46.7	103	5.36
100	♀	47	5.1	1050	1.12	4.01	96.6	3.58	0.042	22.3	79.5	1.91	11.1	39.7	94.8	4.98
200	♂	80	8.1	1270	1.44	6.65	195	4.59	0.034	17.7	81.5	2.41	11.4*	52.5	154	6.37
200	♀	103	9.3	1460	1.62	6.71	210	4.13	0.032	17.4	72.1	2.26	11.3	46.5	145	6.37
340	♂	182	12.0	1790	2.02	9.21	276	4.62	0.033	16.8	77.5	2.31	11.4	52.5	155	6.70
Variation coefficient (standard deviation calculated in per cent of mean)																
2.5	—	6.1	7.0	9.4	9.4	5.7	11.0	11.0**	12.4**	8.9	5.3	8.0	2.4	9.3	13.2	10.3

* Slight but insignificant difference from the value pulbished previously (9) is due to substitution of one individual with another.

** Calculated from variation coefficient of PNA-P/liver and DNA-P/liver or P-N/liver.

nuclei in the total liver nuclei was found to be practically constant in all the growth stages examined, clustering around 65 per cent.*

DNA, PNA, and protein contents of the liver are expressed in three ways: quantity per liver, concentration (quantity per 100 g. fresh tissue), and quantity per average cell. In Table III are given also per cent standard deviations, or variation coefficients (Vc), of individual determinations. It should be noted that among the Vc's indicated, the Vc of DNA per cell stands by far the lowest, being just comparable to that of the body weight. Further, the Vc's of PNA per liver and PNA per 100 g. liver are considerably lower than the corresponding ones of DNA. Lower Vc's of PNA are also found in the data of Klein *et al.* (*15*). As far as PNA and protein are concerned, the expression in quantity per 100 g. liver seems to give the lowest magnitude of variation, and that in cell unit is subject to the greatest variation. As shown in Table II, variation of individual figures is largely not due to experimental error but rather due to variability among animals.

The growth of the liver, which becomes slower in the adult but does not stop in males at least up to body weight of 340 g., is the result of both cell growth and cell proliferation, but the contribution of the latter is much greater than the former. From body weight of 12 g. to that of 340 g., DNA per liver increases to a lesser extent than the liver weight except for between 25 and 50 g. (Fig. 1). Consequently, DNA per 100 g. liver decreases progressively, and beyond body weight of 100 g. there is practically no increase of DNA per cell. PNA and protein per liver increase hand in hand, and unlike to the case of DNA, the extent of over-all increase of these substances exceeds that of the liver weight. In the case of PNA, this is exclusively due to a tremendously high rate of increase between 25 and 50 g. (Fig. 1), in other stages the rate of PNA increase being rather slightly lower than that of the liver weight increase. Thus, the over-all situation of PNA increase proves to be similar to DNA increase. In contrast to this, the rate of protein increase relative to the liver growth rate is steady throughout the course of the postnatal growth.

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* The average cell mass of the liver is thus merely a statistical mean value of 65 per cent parenchymal cells and 35 per cent other cell types (mostly Kupffer cells). In volume, however, parenchymal cells occupy about 90 per cent of the total liver, as determined microscopically by Chalkley method (*16*). Assuming that specific gravities of various cell types are substantially identical, the figure of the average cell mass may be taken as the mass of the average parenchymal cell in the first approximation.

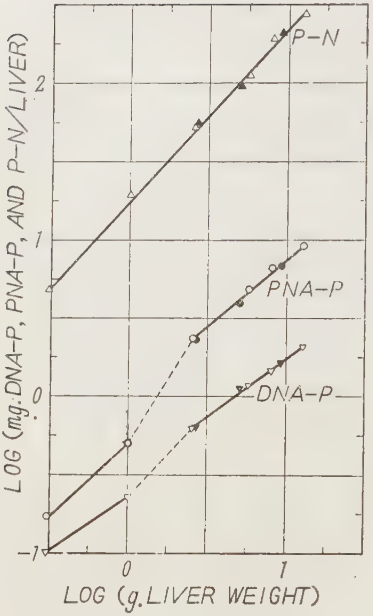

FIG. 1. Rate of increase of DNA-P, PNA-P, and protein-N in the liver relative to the growth rate of the organ throughout the course of the postnatal growth of the rat. Solid marks: female. Spaced marks: mixed sexes or male.

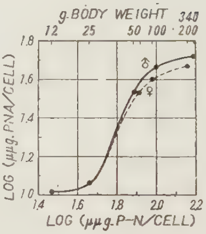

FIG. 2. Rate of increase of PNA-P per cell relative to that of protein-N per cell in the postnatal growth of the rat liver.

The above-noted steep increase of PNA in rapidly growing rats is reflected by the concentration of PNA (per 100 g. liver) which reaches the highest value at 50 g. stage. PNA and protein in cell unit increase also up to 200 g. body weight stage, but, as is seen from S-shaped allometric plot of Fig. 2, rates of their increase do not parallel to each other. Between 25 and 50 g. stages PNA per cell increases to a much higher extent than protein per cell, but in most of the other stages the rate of protein increase definitely exceeds that of PNA increase. The same holds also for PNA and protein per liver. Thus, the ratio PNA-P/DNA-P increases progressively during the course of the postnatal growth, while the ratio PNA-P/P-N has a peak in 50–100 g. stages.

The same situation is more impressively demonstrated in Fig. 3, which illustrates the increase of protein, PNA, and DNA per liver during the whole period of postnatal growth. Along abscissa is plotted the average age of animals. Although the rate of the body weight increase may vary rather considerably among different animals, the rats used in this experiment were altogether retained under good dietary conditions and grew rapidly, so that the growth of these animals may

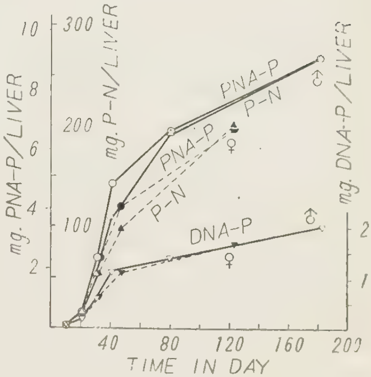

Fig. 3. Increase of DNA-P, PNA-P, and protein-N of the rat liver during the course of the postnatal growth. Solid lines: male or mixed sexes. Broken lines: female.

be taken as typical one.* From Fig. 3 it is clear that the extent of the over-all increase of PNA is quite similar to that of protein, but remarkably greater than that of DNA. The increase of PNA is closely paralleled by that of protein in earlier and later period of postnatal growth, but between 25 and 100 g. stages PNA rises more steeply and reaches a higher level than protein. Such a lagging of the protein increase behind the PNA increase may probably be not independent of the rapid growth of the liver as well as of the whole animal, which begins after weaning and ceases before puberty. In general, females grow more slowly than males, but the pattern of the liver growth is the same in both sexes, as far as protein and nucleic acids are concerned.

From what has been said above it is clear that the growth of the liver and the increase of PNA and protein are most rapid in 25–100 g. body weight stages. The increase of DNA also is not the exception to this rule. The rate of increase of liver DNA is highest between body weights of 25 and 50 g. (9 per cent per day in the average). It should be pointed out here that the polyploidization of the parenchymal nuclei occurs most extensively in this period (18). In adult male rats (above 200 g.) the rate of DNA increase is the lowest (0.3 per cent per day).

Additional experiments were conducted with adult rats rendered dying from prolonged starvation and with young rats whose growth was severely delayed by restricted calorie intake (Table IV). Comparison with normal animals was made in the experiment of fasting at the level of initial body weight (200 g.) and in the experiment of growth retardation at the level of final body weight (50 g.). In both cases,

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* Microscopic observations showed that livers examined in this study altogether have abundant basophilic clumps in the cytoplasm of hepatic cells (type I of the classification of Sibatani (*17*)), which are characteristic of normal rats retained under good nutritional conditions.

TABLE IV

Changes in Cell Number and DNA, PNA, and Protein Contents of the Liver Caused by Growth Inhibition of Young Rats and Prolonged Starvation of Adult Rats

(Average of three animals each)

	Initial body weight	Final body weight	Period of expt.	Per cent of control						
				body weight	liver weight	DNA per cell	cell number	Quatity per liver		
								DNA-P	PNA-P	P-N
	g.	g.	days							
Grow inhibition	38	50	28	100	70	101	98	99	65	78
Prolonged starvation	200	136	9	66	47	98	102	99	35	44

DNA per liver and per cell, and hence cell number, showed no differences from control values, while liver weight, PNA and protein N per liver, and PNA-P/P-N decreased significantly, the diminution, of course, bring more extensive in prolonged starvation. Here it should be noted that PNA is affected more severely than protein under unfavorable dietary conditions. Indeed, in delayed growth PNA increase is depressed to a greater extent than protein increase, so that the latter may no longer be preceded by the former, as is the case in normally growing young rats. This is mirrored by a lowered value of the ratio PNA-P/P-N (0.036 against 0.042 found in control rats). Accordingly, it, may be generalized that in the liver rapid accumulation or loss of protein lags behind the corresponding rise or fall of PNA.

DISCUSSION

In the experiments described in this paper, number of nuclei per liver was estimated from determinations of DNA contents of the whole liver and of average nuclei. One might wonder if such an indirect method involved error of rather large magnitude. Actually, however, the error proved to be relatively small, as indicated in Tables I and II. In most of the determinations involved in our experiment, the magnitude of variability among animals within one and the same group proved to be far larger than that of the experimental error estimated. Here it should be emphasized that experimental errors given in Tables I and II are exceedingly small if one compares them with errors presented by Klein *et al.* (*15*) in experiments of mouse ascites tumor. This may partly be due to the method of determining DNA, for the Swedish workers have employed the Schmidt-Thannhauser method (*19*), which they have stated causes much error in the determination of DNA. However, error of cell or nuclear count in the experiments of Klein *et al.* (6.8 per cent) is also very much larger than that found in our method (1.1–1.8 per cent). The latter may have resulted from completely homogeneous distribution of nuclei in our samples used for counting nuclei (see also foot note on page 96).

It is noteworthy that the variability among animals of DNA per nucleus is very small. This proves that at a given growth stage average DNA content of liver nuclei is far more stable than DNA and cell number per liver, or DNA and PNA per 100 g. liver. Indeed, quantity of DNA per liver, which Mandel *et al.* (*20*) have used for the reference standard of biochemical determinations, was found to be more variable than PNA per 100 g. liver, as far as normal healthy animals are concerned.

As regards the average DNA content of liver nuclei, discussion was given elsewhere (*9*). Here some additional remarks should be made with respect to discrepancies among the data of different investigators. Our values obtained with suckling rats (12 and 25 g.) are of the same order as is accepted generally to be characteristic of mammalian diploid nuclei (*21*, *22*, *23*). As to the adult rats, our values are close to those reported by Dounce *et al.* (*24*) and Campbell and Kosterlitz (*25*) (11 μμg.), but slightly higher than those of Price and Laird (*4*), Harrison (*26*), or Ely and Ross (*27*) (normal rats) (10 μμg.). It is curious that C. Vendrely (*23*) repeatedly obtained significantly lower values (8 μμg.), and that those of Cunningham *et al.* (*8*), whose method we have used here with some modification, are exceedingly low (6 μμg.), being rather comparable to our values for 12 g. suckling rats. Values given by Rose *et al.* (*28*) (15 μμg.) are considerably higher than ours and may presumably be erroneous. The same would hold with data of Price *et al.* (*10*) (12–14 μμg.). Mirsky and Kurnick (*29*) have obtained a value of 8 μμg. for both liver and kidney of rats. The correctness of their data may be questioned, because, unlike to rat liver, rat kidney contains little polyploid nuclei (*7*, *18*, *26*). Indeed, we have obtained a value of 6.05 μμg. for kidneys of an adult rat (300 g.). This value is close to the corresponding ones of Vendrely (*23*) and of Harrison (*26*), but much lower than that of Kurnick* (*3*).

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* Some of these disagreements might be due to accidental errors in working out absolute values, but relative values to be obtained across various biological materials would have to show an essential agreement among data from different sources. Actually, however, relative values for liver and kidney of adult rats do not quite agree among different workers including ourselves.

As have been suggested by several authors (*20, 22, 29*), DNA per liver, cell number, and DNA per nucleus are not affected by starvation or malnutrition, being determined uniquely by the maximal body which animals have once attained. Another example for this regularity was obtained with guiean pigs dying from scurvy (*30*). Meanwhile, some investigators are claiming to have proved that DNA per nucleus of the rat liver is affected by metabolic disturbances (*27, 31*), but they have not compared values of DNA content or cell number of the whole liver in experimental animals with those in controls to be chosen appropriately.

The fact that DNA of approximately non-growing tissues such as liver and kidney of adult animals is not affected by unfavorable dietary conditions (*20*) is frequently taken as an evidence suggesting that DNA is an essential component of the gene (23, 32, 33), but the question how such a situation can be realized has ever met no serious investigation both theoretical and experimental. It is true that DNA *in situ* resists various physical means or autolysis more strongly than PNA (*34–38*). However, purine moieties of DNA in the rat liver are reported to be turned over almost as rapidly as those of PNA (*39*). If the same situation be occurring in fasted livers, it should be assumed that DNA be retained in the liver " actively " until fatal death. Here it should be recalled that Racker (*40*) has recently shown that the surgar moiety of DNA, desoxyribose, can be regarded as a potential source of energy in the cell. Therefore, it is quite surprising from the biochemical, if not genetic, standpoint that even in prolonged starvation the nucleic acid that is to be affected is represented by PNA only, DNA thus remaining untouched. In this connection, it appears suggestive that fasted animals die before PNA of the liver is exhausted completely. It might therefore seem that PNA competes with DNA for decomposition resulting in free energy liberation in the cell, and the mechanism involved in such a hypothetical cell management might be sought in a fact that both ribonuclease and desoxyribonuclease of the liver are distributed mainly in the cytoplasm, and not in the nucleus (*41*).

Comparison of our data with those published by Campbell and Kosterlitz (*5*) reveals several discrepancies. As to the regressions of DNA-P per liver of the rat on body weight, our figures on immature rats agree very well with the corresponding line of their regressions, whereas our figures on mature rats deviate significantly from theirs towards lower DNA values (Fig. 4). Such a disagreement may be due to the difference of the strains employed, because it was shown by

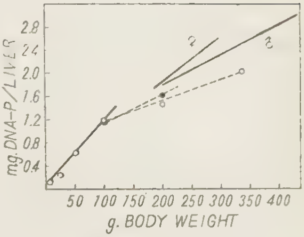

FIG. 4. Comparison of regressions of mg. DNA-P per liver on body weight presented by Campbell and Kosterlitz (5) with our data. Solid lines are regressions given by Campbell and Kosterlitz; broken lines are drawn to fit our values which are indicated with spaced circles (male or mixed sexes) and solid circles (female).

the Scotch authors as well as by ourselves that DNA per liver is determined by body weight and not by dietary conditions. Further it should be pointed out that our data do not seem to quite fit other regressions presented by Campbell and Kosterlitz (*5*) with respect to protein and PNA contents of the liver. Reasons of such discrepancies are not clear.

There seems to be a certain relationship between the change in PNA content and that in protein content of the liver. Unlike to DNA, PNA of the liver shows over-all changes in quantity which parallel those of the liver protein, but PNA changes more sensitively than protein. The accumulation or the loss of PNA seems to take place in higher rate than that of protein, whenever the latter proceeds so rapidly that no secondary changes obscure the relationship. These findings are in unison with observations of some workers on early lag phase of bacterial growth (*42*), regeneration of the liver (43), or development of silk glands in *Bombyx mori* (*44*). However, such a correlation between protein and PNA contents apparently does not hold in some other cases such as scorbutic guinea pig livers (*30*), brains of protein-deficient rats (*20*), or pure cultures of flagellate Protozoa, *Polytomella coeca* (*45*). Further it should not be neglected that PNA of liver mitochrondria is not affected by protein deficiency (*46*).

In this place it is not pertinent to draw any conclusion from our data as to whether some quantitative relation does exist between the PNA and protein syntheses, for, if we could neglect the turnover of proteins in the liver, increment of liver protein in a given length of time represents only a fraction of the quantity of proteins synthesized during the same period in the liver, which produces serum albumin and probably some other sorts of plasma proteins (*47*). Likewise, increment of liver PNA does not necessarily indicate the quantity of PNA newly synthesized, because this also is known to be in a dynamic state (*33*, *39*, *48*).

It is interesting, however, to note that unlike to our case of rat livers, the over-all increase of PNA in several tissues of developing chick embryo is considerably lower than that of protein (*1*). This might be due to the fact that liver is a powerful protein-producing organ, while embryonic tissues may synthesize proteins for their own growth only. It appears, however, that the situation is by no means so simple, because in the course of protein accumulation in silk glands, PNA increases to the same extent as that of the total protein production (*44*).

SUMMARY

Evolution of DNA, PNA, and protein contents and of cell number of rat livers in postnatal growth was investigated. Biochemical determinations were expressed also in cell unit on the basis of cell number, which in turn was estimated from similataneous determinations of DNA per liver and per average nucleus for each specimen examined. It was shown that increases of both cell mass and cell number contribute to the liver growth throughout the course of the postnatal growth. The most rapid growth of the liver occurs between body weights of 25 and 100 g., accompanied by a steep increase of protein and nucleic acids per organ. DNA per nucleus and per liver, and hence the cell number, are irreversibly determined by the maximal body weight which animals have once attained, irrespective of dietary conditions. When rapid accumulation or loss of both PNA and protein is observed in the liver, the change in protein content appreciably lags behind the corresponding change in PNA content.

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The Journal of Biochemistry, Vol. 40, No. 2.

AN IONOGRAPHIC ENRICHMENT OF AN ACTH PREPARATION

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INTRODUCTION

The objective of this investigation was to determine whether the biological activity of an ACTH preparation of low potency could be enriched by means of an ionographic fractionation. In this separation process, strips of filter paper, moistened with a buffer solution of known ionic strength and pH, serve as paths along which ions or other charged particles migrate under the influence of a potential gradient (*3–7*).

MATERIALS AND METHODS

A simplified schematic diagram of the apparatus used is shown in Fig. 1. It consisted essentially of 3 component parts: (1) a removable Lucite frame, A, across which 7 strips of filter paper (Eaton-Dikeman, No. 613, 8 mm. wide) could be stretched. The strips of paper were held taut in place by draw-bolts, B, actuated by stainless steel coil springs at both ends of the frame; the frame itself was adjusted to a horizontal position through the use of a small spirit level; (2) two buffer vessels, C, consisting of glass trays each hoding about one liter of buffer solution. The ends of the paper strips, D, dipped into the buffer solution. The frame, the buffer vessels, and the additional shallow glass vessel, E, (filled with distilled water to help establish equilibrium as regards moisture) were placed inside a water-tight container, F, (12×40×3.5 inches) over which was placed a cover, G. The third component part of the apparatus (3) consisted of 2 electrode vessels, H, connected to the buffer vessels by agar-KCl salt bridges, J, through openings in the cover. Three per cent agar in a 1.5 *M* KCl solution was used in the bridges. The electrodes, K, were made of platinum. The liquid surfaces in the 2 buffer vessels were maintained at the same level throughout all the experiments. In the later phases of the work the ionographic fractionations were made, with identical results, employing the Precision Ionograph,* which recently has been come available (*7*).

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* Reuben Myron Strong Research Fellow, 1951–52.

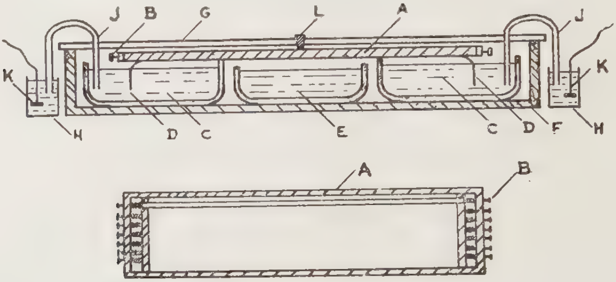

Fig. 1. Schematic diagram of the ionographic apparatus utilized to fractionate the ACTH preparation.

The ACTH preparation had been prepared from pig pituitaries by the acid-acetone extraction method of Lyons (*2*) and further purified by treatment with 9 per cent ammonium hydroxide solution and fractional acetone precipitation. The final material was freeze-dried from aqueous solution. It was one half as active as Armour's LA la standard, that is, its potency was 0.5 U.S.P. unit per mg. A modified brom phenol blue test was used for the detection of the material on the filter paper. In this procedure, the ionogram was dried in a stream of hot air and passed through a saturated alcoholic mercuric chloride solution containing 1 g. of brom phenol blue per 100 ml. of solution. The ionogram was re-dried in a stream of hot air and passed successively through several beakers containing saturated aqueous mercuric chloride solution until all the excess indicator was washed out of the filter paper strip. The ionogram was again dried and the protein zone appeared as a dull green area. It changed to a sharp deep blue zone on passing the ionogram through the vapor from concentrated ammonium hydroxide.

The ionographic fractionation procedures were as follows: All the vessels were filled with the desired veronal buffer made from stock solution prepared in the manner described by Michaelis (*8*) and the filter paper strips were saturated with this buffer solution by means of a pipette, about 2 ml. being required for 100 cm. length of filter paper.

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* Manufactured by Precision Scientific Company, Chicago 47, Illinois.

A potential of the desired voltage was applied across the ends of the paper strips and the instrument was allowed to stand 15 minutes for temperature, voltage and atmosphere to reach a state of equilibrium. From this time on, throughout the course of an experiment, the current (usual range, 75–100 micro-amperes per strip) and voltage readings remained constant. The potential was supplied by a variable-voltage vacuum-tube rectifier, operating from the 110 volt AC line. The aqueous solution of the ACTH preparation was then applied, by means of a micropipette, as a band (about 5–8 mm. in width and containing about 1 mg. of the ACTH preparation) at one or more previously pencil-marked intermediate lines. The ionograph was allowed to run for 3 hours. At the end of that time, the filter paper strips, or ionograms, were dried rapidly by a jet of warm air or by placing them in a constant-temperature oven set 105°.

Another set of experiments was carried out to determine the relation between the percent biological activity and the percent input of total nitrogen for each fraction. Conditions for the experiment were the same as before, that is, a veronal buffer at a pH of 5.5 with an ionic strength of 0.015; in an atmosphere of helium saturated with water vapor; and for a time of 3 hours, at a temperature of 24–26°. The migrant solution was prepared by dissolving ACTH in buffer solution up to a concentration of 60 mg./ml. Aliquots were placed on 20 strips. Some of them were run on the ionograph and some were stored under refrigeration. Of the 14 strips run on the ionograph, two were developed with brom phenol blue to determine the position of the fractions and the corresponding sections of the remaining strips were cut out.

Micro-Kjeldhal determinations for nitrogen were run on half of these fractions, and on 4 of the original strips which were not run on the ionograph. The nitrogen determinations were carried out in two different ways. In one procedure, the fraction, along with the paper strips on which it was present, was digested and a correction was made for the nitrogen present in the paper. In the other procedure, the ACTH fractions were eluted from the paper strips with normal physiological saline for a period of 2 hours at room temperature, and the nitrogen determinations were run on the resulting solution. This latter method of elution was also used when determining the biological activity of the fractions.

The other half of the fractions which were run on the ionograph and two of the original strips were analyzed for their biological activity, using the adrenal ascorbic acid depletion test (*10*).

RESULTS AND DISCUSSION

In Fig. 2 is shown a typical schematic representation of a developed ionogram of the ACTH preparation, indicating separation into 3 fractions. The experimental conditions were: a veronal buffer of an ionic strength of 0.015 and a pH of 5.5; a water-saturated helium atmosphere; time of 3 hours; temperature of 24–26°. Fraction A, which stained heavily, moved to the negative pole; fraction C, which stained lightly, moved to the positive pole; fraction B stained heavily, but failed to move. On elution with normal physiological saline, fraction A was found to contain 80–98 per cent of the biological activity but only 30 per cent of the ACTH preparation, as determined by nitrogen; fraction B contained 2–20 per cent of the activity but 13 per cent of the ACTH preparation. Fraction C exhibited no activity although it contained 40 per cent of the ACTH preparation. It is apparent, therefore, that most of the biological activity of the ACTH preparation was associated with only one-third of the preparation, as determined on the basis of nitrogen content.

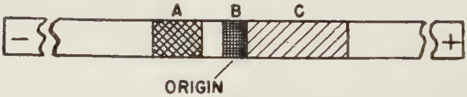

FIG. 2. A typical ionogram of an ACTH preparation; veronal buffer, pH 5.5; ionic strength, 0.015; potential gradient 6 volts/cm.; time, 3 hrs.; temperature, 24–26°; water-saturated helium atmosphere. Fraction A contained 80–98 per cent of the biological activity; Fraction B, 2–20 per cent; Fraction C, no activity.

The isoelectric point range for each fraction was determined by varying the pH of the buffer solution. At a pH of 6.0–6.6 the mobility of fraction A approached zero; at a pH of 5.0–6.0 the mobility of of fraction B approached zero and at a pH of 4.2–4.8 the mobility of fraction C approached zero. On this basis, the isoelectric region of fraction A can be given as 6.0–6.6; that of fraction B as 5.0–6.0, and fraction C, 4.2–4.8. No correction for possible shift of the ioselectric point due to electroosmosis was made in these determinations, since the experiments indicated only that it existed within a pH " range " rather than as a " point " value. Under the conditions of the experiment, the effect of electroosmosis on the isoelectric point has been found to be small in most cases so far investigated (*7*).

Sayers *et al.* (*9*) and Li *et al.* (*1*) reported that the active fraction when studied on the classical electrophoretic apparatus had an isoelectric point in the range of 4.6–4.7. However, from the ionographic experiments reported here, the active fraction appeared to have a much higher isoelectric point and the fraction with an isoelectric point in the region of 4.6–4.7 contained no activity. The higher isoelectric point range for the active fraction reported here is in line with more recent reports by Lesh *et al* (*11*) and Li *et al.* (*12*, *13*) who now place the isoelectric point of the active fraction in the region of pH =9.

The active fraction, A, on the developed ionogram appeared as a zone usually about twice the width of the initial zone of application. The spreading that occurred, may be due in part to adsorption of the migrant by the paper strip, and in part to the probability that this fraction is heterogeneous in nature and composed, perhaps, of a large number of electrophoretically similar but not identical substances.

TABLE I

Comparative Properties of Original and Fractionated ACTH Preparation

	Whole ACTH preparation	Fraction A	Fraction B	Fraction C
Charge on migrant at pH 5.5		+	0	—
Iosoelectric point range		6.0—6.6	5.0—6.0	4.2—4.7
ACTH preparation contained in each fraction, *mg.*	1.22	.38	.26	.55
ACTH preparation eluted, *mg.*	1.03	.37	.15	.49
Biological activity, *per cent*	·	80—98	2—20	0

In Table I, the amount of total nitrogen corrected for the filter paper nitrogen is shown for each fraction; also shown is the amount of eluted nitrogen. It should be noted that the ACTH fractions on the ionogram were not removed completely by elution with normal physiological saline due, perhaps, to some small amount of adsorption of the protein on the filter paper strips at a pH of 5.5. In general, a protein will be increasingly adsorbed as it is displaced to the acid side of its isoelectric point (*7*). However, the main cause of adsorption in this case was that the paper strips were dried out in warm air before elution.

SUMMARY

An ACTH preparation of low potency prepared from pig pituitaries was fractionated ionographically, with the result that the active material was separated out, largely into one well-defined zone. Approximately one milligram of the ACTH preparation was required for a single run. The experimental conditions were: a veronal buffer of ionic strength 0.015 at pH 5.5 to saturate the filter paper strips across which was applied a potential gradient of 6 volts per cm. for 3 hours. The strips were held in a horizontal position in a water-staurated helium atmosphere, at a temperature of 24–26°. The ACTH preparation separated into 3 fractions. One of these contained 80–98 per cent of the biological activity and 30 per cent of the starting material. It bore a net positive charge and had an isoelectric point range of 6.0-6.6. A second fraction which contained 2–20 per cent of the activity and 13 per cent of the starting material, failed to undergo electromigration under the conditions of the experiment. The third fraction, which bore a net negative charge, exhibited no biological activity although it contained 40 per cent of the starting material.

ACKNOWLEDGEMENTS

The ACTH preparation (control No. XI-134–3) was supplied by G. D. Searle and Co., Skokie, Illinois. We are indebted to Dr. F. J. Saunders of G. D. Searle and Co., for the determination of biological activity of the ACTH preparation.

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The Journal of Biochemistry, Vol. 40.,No. 2.

FEULGEN REACTION AND QUANTITATIVE CYTOCHEMISTRY OF DESOXYPENTOSE NUCLEIC ACID*.

III. EFFECTS OF HISTONE ON THE FEULGEN REACTION IN VITRO

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(Received for publication, November 21, 1952)

In previous communications (*1*, *2*) it has been pointed out that the intensity of the Feulgen reaction *in vitro* is considerably modified by the presence of histone and other proteins in the color-developing mixture. This finding is compatible with classical investigations of Widström (*3*) and Caspersson (*4*) on applying the Feulgen reaction to quantitative determination of DNA, which they state is affected by co-existing protein. Besides DNA, cell nuclei contain proteins the quantity of which, in contrast to DNA, is very much dependent on the physiological activity of cells (*5*, *6*). Consequently we can not *a priori* expect that the Feulgen reaction *in situ* may be taken as a measure of the relative DNA content with a variety of cell nuclei (*7*, *8*). In order to establish how far proteins in cell nuclei can affect the intensity of the histochemical Feulgen reaction, it would be required to study the nature and the extent of the protein effect on the Feulgen reaction *in vitro* as well as *in situ*. As long as these questions are not answered adequately, it could not be excluded that any data on the DNA content of cell nuclei, as obtained by the *correct* microspectrophotometry (*9*, *10*) of Feulgen-stained materials, reflect not only the quantity of DNA, but also the type and the relative abundance to DNA of nuclear proteins. In this paper the author presents the results of investigation concerning the effect of histone on the Feulgen reaction *in vitro*.

The color intensity and the absorption curve of the Feulgen reaction are affected by a number of factors involved in the reaction procedure. The effect of each of these factors was evaluated in a previous communication (*11*). The effect of histone has now been investigated in combination with changes in these factors.

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* I. Estimation of the loss of tissue DNA caused by fixation and acid hydrolysis (*12*). II. Factors affecting the Feulgen reaction *in vitro* (*11*). Preliminary reports were published (*23*, *24*) as parts of more extensive investigations.

According to the previous observation of the author (*1*, *2*), the intensity of the Feulgen reaction is remarkably enhanced by the addition of histone when the color is allowed to develop in a markedly acidic medium (0.05 *N* HCl). Although this has been confirmed by subsequent experiments, it has further been revealed that the effect of histone on the Feulgen reaction is far from simple. Histone can act to augment or to diminish the color intensity of the Feulgen reaction according to the conditions of the hydrolysis and the color development. Furthermore, the spectral absorption maximum of the pigment formed is shifted towards longer wavelengths by the addition of histone.

MATERIALS AND METHODS*

Materials used and experimental conditions are the same as in the previous reports (*11*, *12*), but some additional remarks may be necessary. DNH and histone were extracted from isolated nuclei of beef spleen and calf thymus, respectively, essentially in accordance with the method of Mirsky and Pollister (*13*). Hydrolysis was conducted in *N* HCl at 60° as described before (*11*) or in 0.05 *N* HCl at 100°, mostly using 0.04 per cent aqueous DNA (Na salt). In the latter case DNA was hydrolyzed with equal volume of 0.1 *N* HCl in a boiling water bath. After cooling, 1.5 volumes of 0.1 *M* glycine in 0.1 *N* NaCl (called below " glycine-NaCl ") were added to the hydrolysate to make the pH of the solution 2.28. Histone was dissolved (about 0.07 per cent or higher) in 0.1 *N* HCl or Sörensen's 0.1 *M* glycine buffer at pH 2.28 consisting of 2 volumes of 0.1 *N* HCl and 3 volumes of glycine-NaCl. It was added to DNA either before or after hydrolysis: in some earlier experiments aqueous DNA was mixed with equal volume of the histone solution in 0.1 *N* HCl and then hydrolyzed; but later the ordinary hydrolysate was mixed with histone solution; when necessary, the pH was adjusted to 2.28 by appropriate addition of glycine-NaCl, in order that the standard procedure of the color development might be followed. Between the results obtained by the two methods of adding histone to DNA, there was only a slight difference which could by no means be taken as essential.

The standard procedure of the color development presented in a previous paper (*11*) of this series was employed except for modifications indicated. This consists in adjusting the pH of the reaction mixture to 2.28 in the presence of excess SO_2 and with 20-fold dilution of FSA. To neutralized or buffered hydrolysate of DNA (2 ml.) containing about 20 μg. phosphorus were added 0.1 *M* glycine buffer (pH 2.28) up to 8.5 ml., then 1 ml. MBS and 0.5 ml. FSA. The tube was immediately stoppered and the content was mixed thoroughly. After standing at room temperature for 3–4 hours, the color intensity was measured against a blank without DNA which remained colorless or against distilled water with Beckman spectrophotometer at 546 and 580 mμ. The extinction values (E) to be given in this paper always refer to 1 cm. light path and to 1 μg. DNA-P per ml. of the final reaction mixture. These values fluctuated to a certain extent from experiment to experiment, but were proved to be reproducible with tests run simultaneously. A part of the fluctuation of these values presented in this paper may have been due to slight deviations from the expected values of DNA concentration, precise determination of which was not made in individual tests, because only relative values were necessary in most of the experiments made.

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* The following abbreviations are used: DNA, desoxypentose nucleic acid; DNH, desoxypentose nucleohistone; MBS, aqueous solution of sodium metabisufite (15 per cent); FSA, Schiff reagent or fuchsin-sulfurous acid.

RESULTS

Precipitability of Hydrolyzed DNA with Histone—If a moderate quantity of histone is added to partially acid-hydrolyzed DNA dissolved in a weakly acidic medium, a precipitate is formed. The degree of precipitation of DNA can approximately be estimated by the Feulgen reaction of the supernate, although the intensity of the reaction is affected by the co-existing histone. The precipitate is made soluble again by a further addition of histone. The precipitability of DNA is diminished also by prolongation of the hydrolysis. This is true for DNA hydrolyzed both in 0.05 N HCl at 100° and in N HCl at 60°. Thus, in case of hydrolysis for 2 minutes in 0.05 N HCl maximal precipitation is observed when ratio of the quantity of histone to that of DNA (before hydrolysis) is about 0.7; if this ratio is higher than 3, practically no precipitation is observed under the same conditions of hydrolysis. When the ratio is about 1.4, an appreciable amount of precipitate is formed after hydrolysis for 3 minutes, but not for 5 minutes. With N HCl at 60°, hydrolysis for 30 minutes and histone/DNA ratio of 3.5 gives no precipitate, while hydrolysis for 15 minutes with the same histone/DNA ratio results in the formation of a small amount of precipitate. When the histone/DNA ratio is raised to 7, appearance of precipitate is inhibited even with hydrolysis for 15 minutes. It was also found that acid-precipitated DNH which goes into solution only partially during the course of the hydrolysis could be solubilized to a remarkable extent by the addition of excess histone to the hydrolyzing mixture.

In most experiments described below, conditions were chosen so as to cause no precipitation of hydrolyzed DNA-histone complex, otherwise an accurate measurement of the extinction was impossible.

Shift of the Absorption Maximum of the Feulgen Stain—Absorption maximum of the Feulgen stain produced with DNA alone under optimal conditions for the color development lies at 546 mμ or at somewhat longer wavelengths, small variation being observable in individual experiments (*11*). When histone is added to the reaction mixture prior to the color development, the whole absorption curve of the stain formed shifts towards the longer wavelengths, the maximum now being located at 575–580 mμ. The absorption spectrum of the Feulgen reaction thus obtained closely resembles that of Schiff reaction of formaldehyde developed under comparable conditions (*11*). These curves are shown in Fig. 1. Indeed, the occurrence of such a shift of the absorption maximum is recognizable even with visual inspection: the Feulgen reaction developed with histone and the Schiff reaction of formaldehyde give pigments of more violet tinge than the ordinary Feulgen reaction developed without histone does.

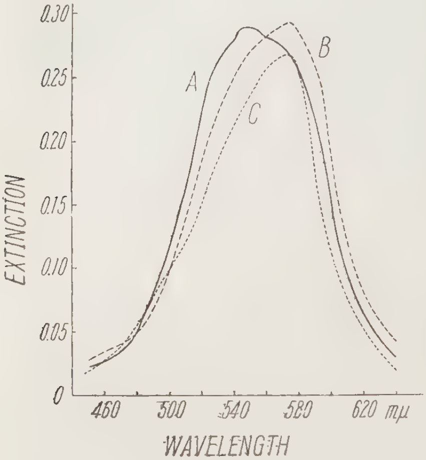

FIG. 1. Absorption spectra of pigments produced by Feulgen reaction of DNA with and without histone added and by Schiff reaction of formaldehyde. A, Feulgen reaction with DNA alone; B, Feulgen reaction with DNA plus histone; C, Schiff reaction. In A and B, hydrolysis in 0.05 *N* HCl at 100° for 1.5 minutes; histone/DNA 7; in C, 1 ml. of strongly diluted commercial formalin was added with pH 2.28 buffer and the color was developed according to the standard procedure of the Feulgen reaction. The W ratio: A 1.12, B 0.92, C 0.84. Extinction values refer to 1 μg. DNA P/ml. of the reaction mixture in A and B, but are in arbitrary unit in C. A weak constriction near the top of curve A is not always to be observed with Feulgen reaction developed with DNA alone.

As suggested in the previous report (*11*), the ratio of extinction values at two wavelengths 546 and 580 mμ may serve as a quantitative measure of the shift of the whole absorption curve. This ratio is called henceforth the "W ratio." W ratio obtained with and without histone added under various conditions resulting in widely different color intensities (*11*) are summarized in Table I. If the Feulgen reaction is conducted under optimal or suboptimal conditions, the W ratio is mostly higher than 1.1 without histone added, but lower than unity under addition of histone. In the latter case, it is close to the W ratio of the Schiff reaction of formalaldehyde developed according to the standard procedure of the Feulgen reaction. However, values of the W ratio fluctuate notably in individual tests. This may partly be due to the temperature at which the reaction is allowed to proceed (*11*). The W ratio is further subjected to variation according to changes of the variables involved in the procedure of the Feulgen reaction (*11*). With all these in mind, the difference between the W ratios with DNA alone

TABLE I

Alteration of the Spectral Absorption Curve of the Feulgen Pigment Effected by the Presence of Histone in the Color Developing Mixture, as Expressed by the W Ratio (E_{546}/E_{580}*)

Hydrolysis medium	Period of hydrolysis	$\frac{\text{Histon}}{\text{DNA}}$ ratio	Conditions of color development	W ratio without histone	W ratio with histone
	minutes	*µg./µg.*			
0.05 *N* HCl 100°	1.5	7	—	1.15	0.96
	2	3.5	—	1.13	0.88
	2	7	—	1.13	0.92
	2	3.5	without MBS**	1.13	0.82
	3	3.5	2 ml. hydrolysate +8 ml. FSA	1.02	0.76
	3	7	—	1.11	0.87
	3	7	without MBS	1.16	0.88
	5	0.9	without MBS	1.07	1.00
	5	1.8	without MBS	1.15	1.00
	5	7	without MBS	1.07	0.96
N HCl 60°	13	14	—	1.08	0.88
	13	14	without MBS	1.08	0.90
	15	7	—	1.14	0.92
	30	7	—	1.12	0.92

* The W ratios included in this and the following tables were calculated from extinctions measured at 546±4 and 580±4 mµ.

** Addition of 1 ml. MBS was replaced with that of 1 ml. glycine buffer of pH 2.28.

and with DNA plus histone is significant in most experiments conducted under various conditions.

Modification of the Intensity of the Feulgen Color—While the shift of the absorption maximum of the Feulgen pigment caused by the presence of histone in the reaction mixture is rather unique under various conditions which modify the intensity of the Feulgen color developed, the situation is entirely different concerning the histone effect on the color intensity of the stain formed. This is because conditions which effect higher intensity of the Feulgen color with DNA alone cause depression of the color development in the presence of histone added, whereas those resulting in lower color intensity with DNA only rather enhance the color development, if histone has been added to the reaction mixture. These are true for conditions concerning both the hydrolysis and the composition of the reaction mixture (*cf.*, *11*). Thus, the addition of histone enhances the color intensity in cases of longer hydrolysis time (over-hydrolysis), of lower pH of the reaction mixture, and of absence of a large excess of MBS or FSA, but lowers it in cases of optimal hydrolysis time and of the presence of large excess of MBS or FSA in the reaction mixture. It must, however, be pointed out that the addition of histone is ineffective in levelling off the different intensities of the Feulgen color resulting from such modifications of hydrolyzing or color developing conditions.

As the measure of the histone effect on the color intensity we may take a ratio of extinctions at a given wavelength obtained with and without histone. This ratio is designated below the "H ratio." The relation between the H ratio and the color intensity with DNA alone realized under various experimental conditions is illustrated in Table II.

Although such a trend as indicated above could be observed invariably in individual experiments, the degree of the augmentation or depression of the color intensity effected by histone was not reproduced quantitatively for reasons which are not clear at present. It is nevertheless probable that the magnitude of the H ratio is modified more severely by the change in the composition of the color developing medium than that of the hydrolysis conditions within the range of our experimentation.

Variables Affecting the Magnitude of Histone Effect—Some variables involved in the Fedulgen reaction under the addition of histone were further studied in order to examine whether some systematic changes were to be observed in the W and H ratios.

TABLE II

Effect of Histone on the Color Intensity of Feulgen Reactions Conducted under Various Conditions as Expressed by the H Ratio (E with Histone/E without Histone).
(Extinctions of different experiments are not corrected on a level comparable with one another)

Exptl. No.	Experimental conditions			E_{546} per μg. DNA P/ml.		H ratio	
	Histone/DNA ratio μg./μg.	Hydrolysis	Color development	without histone	with histone	546 mμ.	580 mμ.
1	7	0.05 *N* HCl 100° 3 min.	—	0.226	0.158	0.70	0.90
			without MBS*	0.130	0.215	1.66	2.14
			0.05 *N* HCl without MBS**	0.016	0.100	6.3	6.6
2	3.5	0.05 *N* HCl 100° 3 min.	2 ml. hydrolysate+8 ml. FSA	0.233	0.093	0.40	0.52
3	3.5	0.05 *N* HCl 100° 2 min.	without MBS	0.221	0.150	0.63	0.95
		5 min.		0.132	0.196	1.48	1.73
		10 min.		0.054	0.097	1.80	1.87
4	7	*N* HCl 60° 30 min.	—	0.293	0.199	0.68	0.80
		60 min.		0.206	0.217	1.05	1.16
		120 min.		0.103	0.132	1.28	1.25

* Addition of 1 ml. MBS was replaced with that of 1 ml. 0.1 *M* glycine buffer of pH 2.28.

** Glycine buffer and MBS were replaced with 0.05 *N* HCl.

Fig. 2 illustrates the course of color development of the Feulgen reactions with and without histone added. From accompanying plots of W and H ratios against time it is obviously seen that neither of the W and H ratios remains at a constant value during the course of the color development. The W ratios with and without histone rise rather rapidly at the beginning of the color development. In two hours they reach more or less stable levels that were stated above to be characteristic of the respective cases. There is also a trend of rising of the H ratio at both wavelengths examined, except for the very early period of color development.

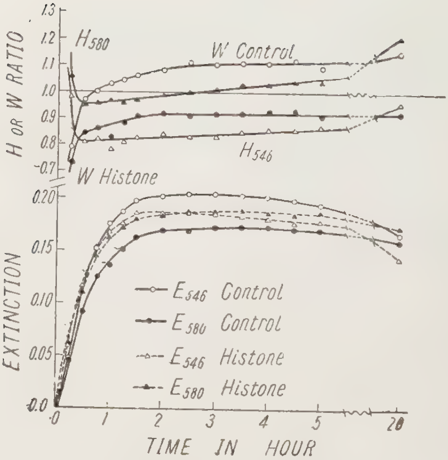

FIG. 2. Progression of color development of the Feulgen reaction with and without histone added and shift of W and H ratios resulted herefrom. Hydrolysis in *N* HCl at 60° for 30 minutes.

While, as shown previously (*11*), the W ratio without histone was lowered by prolonged hydrolysis and becomes less than unity in cases of advanced over-hydrolysis, it was difficult to recognize any definable trend in the change of the W ratio with histone, although this also was subjected to occasional variation.

In another series of experiments effect of histone added to fully recolorized mixture was investigated. Results of a typical experiment are shown in Table III. In this, the color was first allowed to develop according to the standard procedure in a 8 ml. mixture (realized by reducing the volume of the buffer added) in the absence of histone for 2.75 hours; then 2 ml. histone solution in buffer were introduced into this mixture (a). Control tests were run also in 8 ml. (b) with,

TABLE III

Effect of Histone Added after Full Development of the Feulgen Color on the W and H Ratios.

(Hydrolysis in *N* HCl at 60° for 30 mins. Histone/DNA ratio=8. See text for detail of the experiment)

	Extinction per μg. DNA P per ml. final reaction fluid at				W ratio		H ratio at 580 mμ	
	546 mμ		580 mμ					
Time after addition of histone in a)	4 min.	50 min.	4 min.	50 min.	4 min.	50 min.	4 min.	50 min.
(a) Histone added posterior to color development	0.223	0.227	0.212	0.219	1.05	1.04	1.07	1.15
(b) Histone added prior to color development	0.180	0.181	0.193	0.193	0.93	0.94	0.97	1.02
(c) No histone added	0.223	0.216	0.199	0.190	1.12	1.13	—	—

and (c) without, histone added prior to starting the color development; simultaneously with the addition of histone in (a), 2 ml. buffer were added to each of (b) and (c). These were immediately followed by extinction measurements which were continued for 50 minutes at intervals. Addition of histone to the already colored mixture gave rise to an immediate increase of extinction at 580 mμ, that at 546 mμ remaining unchanged, so that at 4 minutes the W ratio of (a) was intermediate between those of (b) and (c). There was little change of the extinctions at both wavelengths in (b) but slight increase in (a) and decrease in (c) during the course of measurements, the W ratios obtained at 4 and 50 minutes showing only an immaterial difference. In contrast, the H ratio, especially at 580 mμ, rose progressively above 1 in (a). Thus, the addition of histone is effective in enhancing the absorption at 580 mμ of the preformed Feulgen pigment almost instantaneously, although the absorption at 546 mμ can not be depressed by the histone added afterwards, unlike to the case where histone is added prior to the start of the color development.

The magnitude of histone effect on the color intensity of the Feulgen reaction depends further upon the histone/DNA ratio. This is clearly seen from Table IV. By changing the histone/DNA ratio with fixed concentration either of DNA or of histone, the color intensity per unit quantity of DNA P showed a systematic alteration (Expts, 1B, 2, and 3); of course, without histone added, this was constant for varying concentrations of DNA (Expt. 1A). The observed change in the color intensity reflects an augmentation of the histone effect; that is, the H ratio is invariably more remote from unity with higher histone/DNA ratio, whether conditions employed are to result in a H ratio higher or lower than unity. In contrast to these cases, changing the concentrations of DNA and histone with fixed histone/DNA ratio causes, at least at 546 mμ, no significant alteration of the extinction per unit quantity of DNA P (Expts. 4 and 5). Thus, in the presence of histone, the Beer-Lambert law fails to hold, unless the relative concentration of histone to DNA remains constant. It should further be noted that in most cases the W ratio is little affected by changing the concentration of DNA and/or histone, with fixed or varied histone/DNA ratios.

Effects of Some Basic and Acidic Substances on the Feulgen Reaction—The foregoing experiments suggest that the effect of histone on the Feulgen reaction is due to its complex formation with hydrolyzed DNA. Of course, control tests run with histone alone produced no pigment. It was further proved that the addition of arginine and other basic amino

TABLE IV

Color Intensities of Feulgen Reactions Conducted under the Addition of Histone in Varying Concentrations, with Fixed and Varied Concentrations of DNA Relative to the Histone Added.

(Conditions of hydrolysis: *N* HCl at 60° for 30 minutes)

Exptl. No.	Addition of MBS	Final concn. of DNA	Final concn. of histone	Histone/DNA ratio	Extinction per μg. DNA P per ml. of reaction mixture at		W ratio	H ratio 546 at mμ
					546 mμ	580 mμ		
		μg./ml.	*μg./ml.*	*μg./μg.*				
1A	+	40	0	0	0.224	0.200	1.12	—
		20	0	0	0.224	0.199	1.12	—
		10	0	0	0.223	0.198	1.13	—
2B	+	40	140	3.5	0.179	0.196	0.91	0.80
		20	140	7	0.171	0.187	0.92	0.76
		10	140	14	0.159	0.174	0.91	0.71
2	—	20	140	7	0.142	0.144	0.99	4.9
		10	140	14	0.149	0.150	0.99	5.1
		5	140	28	0.162	0.162	1.00	5.6
3	+	12.5	200	16	0.176	0.186	0.95	0.74
		12.5	400	32	0.159	0.174	0.91	0.67
		12.5	800	64	0.145	0.159	0.91	0.61
4	+	25	400	16	0.175	0.187	0.94	0.73
		12.5	200	16	0.176	0.186	0.95	0.74
		6.25	100	16	0.177	0.194	0.91	0.74
5	—	20	140	7	0.142	0.144	0.99	4.9
		5	35	7	0.140	0.140	1.00	4.8

acids, non-hydrolyzed DNA, or chondroitin sulfate* to hydrolyzed DNA exerts little if any influence upon the formation of the Feulgen stain, the H and W ratios being practically unchanged from those of controls. Thise findings support the view that the effect of histone is caused by the formation of electrostatic combination in weakly acidic media between DNA phosphorus group and histone as a base of relatively high molecular weight. It seemed thus likely that the addition of chondroitin sulfate along with histone prior to the color development suffices to depress the histone effect on the Feulgen reaction. In fact, Anderson and Wilbur (*14*) have shown that heparin, a close relative of chondroitin sulfate, is effective in liberating DNA from isolated nuclei, thus replacing the latter in the nuclear nucleoprotein complex.

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* Received from Dr. T. Maki, Minophagen Pharmaceutical Co., Tokyo.

Indeed, histone is readily precipitated by adding chondroitin sulfate to the reaction mixture. However, the Feulgen color was developed on the precipitate formed, and the centrifuged supernatant exhibited essentially no coloration. This was the same even with a great excess of chondroitin sulfate (its ratio to DNA was 20:1, histone/DNA was 3.5). With such a system, an accurate measurement of the extinction was impossible, since the stain was formed on the suspended material. However, forcible measurement gave a W ratio of 0.88, which was close to that without chondroitin sulfate added, a value of 0.92. Thus, it seems that partially hydrolyzed DNA was not detached from histone and the histone effect on the Feulgen reaction was not effaced even in the presence of a large amount of chondroitin sulfate, the latter simply entering into combination with histone linked to hydrolyzed DNA to result in the formation of a complex insoluble in weakly acidic media. This seems to be contradictory to the experiment of Anderson and Wilbur (*14*). The reason of this discrepancy is not clear; the quantity of chondroitin sulfate may have been insufficient in our experiment, because histone was added also in excess to DNA.

Inhibition of the Histone Effect on the Feulgen Reaction by Sodium Chloride —The occurrence of the electrostatic combination between histone and hydrolyzed DNA may be questioned from the result of the preceding experiment, but it is supported by an experiment with concentrated NaCl. As shown in Table V, NaCl inhibits the development of the Feulgen color rather severely. The degree of inhibition is approximately linear against the concentration of NaCl, but independent of that of DNA (2 and 4 mg. per cent in the final mixture were tested). Here it should be kept in mind that introduction of larger amounts of NaCl into the reaction mixture is expected to affect the buffering of the latter to a certain extent. The degree of the inhibition is higher at 546 mμ than at 580 mμ, so that the W ratio is lowered gradually with increasing salt concentration. This means probably a slight shift of the absorption peak towards the longer wavelengths. Now the effect of histone is effaced almost completely at the higher concentration of NaCl. Thus, the W ratio with histone added rises progressively with the increase of NaCl concentration, to reach finally a value identical with that obtained

TABLE V

Effect of Sodium Chloride on the Feulgen Reaction Developed with and without Histone

(Hydrolysis in *N* HCL at 60° for 30 minutes; histone/DNA ratio=8)

Concentration of NaCl	Without histone added			With histone added		
	color yield in per cent of control at		W ratio	W ratio	H ratio at	
	546 $m\mu$	580 $m\mu$			546 $m\mu$	580 $m\mu$
M						
0 (control)	100	100	1.14	0.89	0.64	0.82
0.3	89.7	90.6	1.13	0.94	0.79	0.96
0.75	75.8	79.9	1.08	1.00	0.89	0.97
1.5	60.2	64.3	1.06	1.06	0.93	0.98

without histone. The H ratio, which in this case is lower than unity, also rises with the increase of NaCl concentration, and approaches to unity at the higher concentrations of the salt.

DISCUSSION

Nature of the Histone Effect on the Feulgen Reaction—There are two possibilities in which histone is able to affect the light-absorbing characteristics of the Feulgen stain formed in its presence: the Feulgen stain is modified 1) directly by an interaction occurring between histone and fuchsin-sulfurous acid, or 2) indirectly by the mediation of the electrostatic combination of histone with hydrolyzed DNA through the phosphate group of the latter. Experiments illustrated in Tables IV and V strongly support the latter possibility, for the proportion to DNA, and not the concentration, of histone is the determining factor of the magnitude of the histone effect on the intensity of the Feulgen color; in the presence of concentrated NaCl the histone-DNA complex must be dissociated, and this will explain the almost complete inhibition of histone effect on both the intensity and the absorption curve of the Feulgen color. At least a part of the shift of the absorption maximum towards the longer wavelengths caused by histone may be due to the alteration in resonating structure of the stain moiety, representing a kind of metachromasia, as indicated by the immediate rise of extinction at 580 $m\mu$ which is observed upon addition of histone to the solution of preformed stain. However, this could be realized equally well by either of the two possibilities mentioned above.

Here it would be reasonable to suspect that the phosphoric acid group of DNA might exert some influence upon the absorption curve of the pigment formed and/or being formed, because they should be masked by entering into combination with histone added, the state of the hydrolyzed DNA molecule becoming more akin to simple aldehyde such as HCHO in respect of the ionic charge. This idea is supported by the noted similarity of the absorption curves of the Feulgen reaction with histone added and the Schiff reaction of HCHO.

However, an examination of the absorption curves of Schiff or Feulgen pigments given by Overend (*15*) with 2-desoxyribose and desoxyribonucleotides makes it somewhat doubtful that the introduction of the phosphoric acid group into the molecule bearing an aldehyde group has in itself any significant effect upon the absorption curve of the pigment produced. But one must be careful about this, because the conditions of the reaction employed by Overend is not identical with ours. Here it should be recalled that the absorption curve is very much dependent on the reaction procedure and the difference in the W ratio may be obscured under unfavorable conditions (*cf.*, *11*).

The molecular size of the reactive substances (thymic acid polynucleotides) also seems to have some bearing on the light-absorbing characteristics of the pigment to be developed (*16*). The change in the color intensity and the W ratio exhibited by the over-hydrolyzed DNA and their modification in the presence of histone might partly be considered along this line. Furthermore, the fact that the situation realized by the addition of histone can not be simulated by adding basic amino acids to the reaction mixture suggets that the effect of histone as observed here is also due to its larger molecular size.

Quantitative Aspect of the Histochemical Feulgen Reaction—Turning to the main problem of our study, *viz.*, the quantitative nature of the histochemical Feulgen reaction which must be developed under coexistence of various amounts and various sorts of proteins, it must be said that much remains to be done in the experimental approach to this problem. Heretofore we could perform a detailed study, confined to the system *in vitro*, only with histone. There are informations (*17*) that DNA in nuclei is associated not only with histone or protamine, but also with proteins of non-histone type. However, it may not be justified to expect that solely experiments *in vitro* like those described above give sufficient basis for a critical evaluation of the histochemical

Feulgen reaction as a reliable test in quantitative-cytochemical analysis of DNA. This is because the Feulgen reaction performed with solid systems such as fixed biological materials can by no means be taken directly comparable with that developed in the liquid system such as the aqueous solution of DNA, even under appropriate addition of one or more sorts of proteins (*18*, *19*). The recognition of such a situtation dissuaded us from extending our study on the Feulgen reaction *in vitro* to the examination of the effects of different kinds of proteins and other substances. Under these circumstances, the nature and the extent of the protein effect involved in the histochemical Feulgen reaction could be investigated only through working directly with stained tissue materials (*20*).

Concerning the Feulgen reaction *in vitro* with DNA in solution, it has been established through our study that the presence of histone generally causes deviations from the Beer-Lambert law. The ratio of histone to DNA is important in this respect. However, it is not certain that the effect of different proteins is similar; nor can the possibility be ruled out that different proteins exert their influence upon the Feulgen reaction in different ways (*2*). According to the recent investigations of the Mirsky group (*5*) nuclei of different cells contain various amounts of different proteins per unit quantity of DNA. These proteins may well affect the Feulgen reaction *in situ* in some way, which may or may not be similar to that observed *in vitro*. However, it seems also by no means to be excluded that the Feulgen reaction *in situ* is little affected by co-existing proteins in solid state as they are in fixed cells. Thus, the data presented in this paper *per se* can not answer the question of whether the Beer-Lambert law is obeyed by the Feulgen pigment formed within the fixed cell nuclei.

The same would hold in Lessler's experiments with a solid system *in vitro* (*21*), in which he measured the transmittance of formalin-fixed and Feulgen-stained gelatin blocks containing DNA in different concentrations (that is, with different gelatin/DNA ratios) with aid of a microscopic photometer (*22*). There was a deviation from the Beer-Lambert law at higher extinction values (about 0.8), but this also might be due to the failure of the apparatus used in eliminating the Schwarzschild-Villiger effect (*10*). Furthermore, the observed deviation may also well have been brought about by the presence in the reaction system of gelatin which should enter into complex formation with DNA. The occurrence of such a situation can readily be inferred on the basis of our experiments illustrated in Table IV, in which the change in histone/

DNA ratio resulted in a failure of the Feulgen stain to follow the Beer-Lambert law. Thus, in attempting such an experiment, it would be required to examine whether gelatin does not interfere the Feulgen reaction of DNA in such a fashion, or, speaking more generally, whether it has materially no detectable effect upon the quantitative nature of the reaction. But even if these suspicions could be eliminated by an adequate experimentation, there would remain the problem of the Beer-Lambert law concerning the stained nuclei completely unsettled, for the question of whether the proteins of cell nuclei do not invalidate the Beer-Lambert law can still not be answered at all.

From what has been said it would be understood that in the present state of our knowledge much uncertainty must be left behind, about the question of how far we can go with quantitative-cytochemical application of the Feulgen reaction into the reliable determination of the quantity of DNA in cell nuclei and the comparison of the data among different kinds of nuclei or nuclei of the same type but in different physiological activities. In order to solve this difficult problem, we are now working directly with Feulgen-stained tissue materials.

SUMMARY

1. The precipitability of partially hydrolyzed DNA with histone depends upon the extent of hydrolysis and the histone/DNA ratio. Addition of histone in excess to hydrolyzed DNA causes no precipitation.

2. Presence of excess histone in the reaction mixture consistently gives rise to a shift towards the longer wavelengths of the maximal spectral absorption of the Feulgen pigment under conditions optimal or suboptimal to the color development.

3. The color intensity of the Feulgen reaction is strongly modified by the presence of histone in the reaction mixture. Under the conditions favorable to the color development with hydrolyzed DNA alone, histone depresses, and under those unfavorable, it enhances the color intensity, but the intensity is never levelled off. The magnitude of this effect is dependent on the histone/DNA ratio: the higher the ratio, the stronger the effect. Thus the presence of excess histone causes the deviation from the Beer-Lambert law of the Feulgen color.

4. The histone effect is effaced almost completely by concentrated NaCl, suggesting that it is due to the electrostatic combination between hydrolyzed DNA and histone.

5. The bearing of these findings on the problem of the quantitative nature of histochemical Feulgen reaction was discussed.

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ON THE KINETICS OF THE HUMAN BLOOD CHOLINESTERASE

II. THE TEMPERATURE EFFECT UPON CHOLINESTERASE ACTIVITY

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It was reported in the previous paper (*1*) that the process of enzymatic hydrolysis of acetylcholine by human blood cholinesterase can best be explained by a reaction scheme involving the formation of an inactive complex consisting one molecule of enzyme with two molecules of substrate in the range of higher substrate concentrations. The mechanism may be represented by following step reactions:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} ES \qquad (1)$$

$$ES \xrightarrow[k_3]{} E + P \qquad (2)$$

$$ES + S \underset{k_5}{\overset{k_4}{\rightleftarrows}} ESS \qquad (3)$$

where E, cholinesterase, S, acetylcholine, P, reaction product, ESS, inactive enzyme-substrate complex and k_1, k_2,..., rate constants.

In the present paper, it is reported on the temperature effect of cholinesterase activity; this has been investigated hitherto only by Glick (*2*) and Goldstein (*3*). As it will be seen later, a positive entropy change for the formation of the enzyme-substrate complex was found. In connection with the similar finding of Laidler (*4*) in his kinetical study of the enzymatic hydrolysis of urea by urease, the finding of the present author seems to be of extreme interest for the theoretical interpretation of enzyme mechanism in general.

With erythrocyte cholinesterase, further, the dissociation constant of the Eq. (3) was investigated in detail.

EXPERIMENTAL METHOD

The experiments were undertaken with the same procedure as described in the previous paper (*1*) and at the temperature of 15, 20, 25, 30 and 37.5°. In the range of these temperature, no appreciable inactivation of the enzyme activity occurs.

RESULTS AND DISCUSSION

The Effect of Temperature on the Reaction Rate—As it is shown in Fig. 1, the activities of both enzymes, of serum and of erythrocytes, increase almost lineally with temperature. The difference in the activity of both types of enzymes is shown by temperature coefficients, Q_{10} (25–35°), which can be calculated as 1.3 for erythrocyte cholinesterase and 1.5 for serum cholinesterase.

The Effect of Temperature on the Michaelis Constant—As given by the following equation, the Michaelis constant, K_m seems not to be simply accepted as the dissociation constant of the enzyme-substrate complex.

$$K_m = (k_2 + k_3) / k_1 \qquad (4)$$

Thus, the energy value caluculated from log K_m and $1/T$ involves the heat of reaction (ΔH) for the formation of enzyme-substrate complex and the heat of activation (ΔE) in the activating step (*cf.*, Neurath (*5*)).

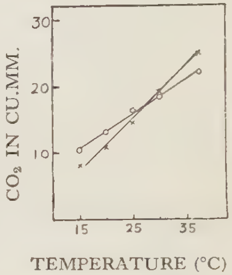

FIG. 1. Effect of temperature upon the activity of both types of cholinesterase; —o—o— erythrocytes; —×—×— serum.

Since the individual rate constant of cholinesterase reaction in each step reaction can not be obtained at present, temperature effect on the Michaelis constant was studied by introducing two presumptions as described in the following sections. Michaelis constants for both types of the enzymes were found to be independent of the temperatures (Table I).

TABLE I

The Values of Michaelis Constants for Both Types Cholinesterase at Different Temperatures

Temperature	K_m, Erythrocyte	K_m, Serum
	mole per lit.	*mole per lit.*
15	4.1×10^{-4}	1.2×10^{-3}
20	3.2× ”	1.2× ”
25	3.3× ”	1.3× ”
30	2.9× ”	1.1× ”
37.5	3.7× ”	1.2× ”

a) In case of $k_3 \ll k_2$:

Since $K_m = k_2/k_1$ from Eq. (4), reciprocal of K_m is to give the equilibrium constant in the Eq. (1).

$$1/K_m = K = k_1/k_2$$

Now, taking ΔH_1 as the heat of the reaction in the ES formation, the following equation is derived from van't Hoff equation.

$$d \log K / d(1/T) = -\Delta H_1 / (2.303 \times R)$$

Since, $\Delta H_1 = 0$ from Table I and

$$\Delta F_1 = -RT \ln K,$$

$$\Delta F_1 = \Delta H_1 - T\Delta S_1,$$

the thermodynamic constants in the reaction of enzyme-substrate complex formation for both types of cholinesterase can be caluculated as given in Table II.

TABLE II

The Thermodynamic Quantities of the Reaction of Eq. (1)

(310.5°K)

	Erythrocyte	Serum
ΔF_1 *(kcal./mole)*	−4.9	−4.2
ΔH_1 *(kcal./mole)*	0	0
ΔS_1 *(cal./mole/deg.)*	+15.8	+13.4

It is notable from the caluculated data that the entropy ΔS_1 is obtained in positive quantity. Recently Laidler and others also have found the positive entropy change in the process of enzyme-substrate complex formation from their thermodynamic analysis on the reaction process of urease and pepsin (*6*). They are in the assumption to explain the above fact that the enzyme molecule itself may change its configuration in forming the stable complex with the substrate, and such changes might consist of an opening out of the enzyme structure. Their results are shown by Table III including those of ours. It is of interest that ΔS_1 of cholinesterase has been obtained somewhat in the same magnitude as that of urease.

b) In case of $k_3 \gg k_2$:

$K_m = k_3/k_1$ from Eq. (4). Therefore, the value of energy ΔE obtained from a plot of log K_m against the reciprocal of absolute temperature is given by

$$\Delta E = \Delta H^* - \Delta H_1^* \quad \ldots\ldots\ldots\ldots\ldots\ldots (5)$$

TABLE III

Summary of Available Kinetic Informations of Enzyme-catalyzed Hydrolysis

Enzyme	Substrate	$\varDelta H$	$\varDelta S$
		kcal.	*cal./mole*
*Urease	Urease	3.3	+13.3
*Pepsin	Carbobenzoxy-L-glutamyl-L-tyrosinester	2.4	+20.6
* "	Carbobenzoxy-L-glutamyl-L-tyrosine	3.0	+24.4
Erythrocyte cholinesterase	Acetylcholine	0	+15.8
Serum cholinesterase	Acetylcholine	0	+13.4

* Their calculations are based upon the assumption that the equilibrium constant is equal to the reciprocal of the Michaelis constant.

in which $\varDelta H^*$ is the heat of activation of $ES \rightarrow ES^*$ and $\varDelta H_1^*$ is that of $E + S \rightarrow ES$.

As $\varDelta E = O$ from experimental results (Table I),

$$\varDelta H^* = \varDelta H_1^*$$

By denoting maximum velocity by V_m and total enzyme by $\sum E$, following equation (6) can be obtained through Eq. (1) and Eq. (2).

$$V_m = k_3 (\sum E) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (6)$$

$$d \log V_m / d (1/T) = d \log k_3 / d (1/T) = -\varDelta H^* / (2.303 \times R)$$

Thus $\varDelta H^*$ can be obtained from a plot of log V_m against 1/T. The plotts of log V_m vs 1/T in two types of cholinesterase are shown in Fig. 2. Thus $\varDelta H^*$ is caluculated as 3.7 *kcal./mole* for erythrocyte cholinesterase and 7.7 *kcal./mole* for serum cholinesterase. The heat of activation $\varDelta H^*$ thus found for the latter is greater than that for the former.

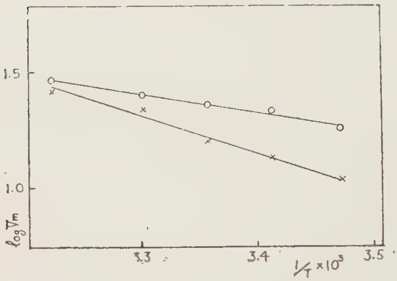

FIG. 2. The relationship log V_m vs. 1/T; —o—o— erythrocytes, —×—×— serum.

The Michaelis constant is thus dealt above under the presumption of $k_3 \ll k_2$ and $k_3 \gg k_2$. The kinetics in the true sense of the reaction mechanism can, however, only be explained after that the real determination of velocity constants in every step reaction will be available.

The Effect of Temperature on Km′, the Dissociation Constant of Inactive Complex ESS—In case of erythrocyte cholinesterase, Eq. (3) interposes in higher substrate concentrations. When the substrate concentration is sufficiently high, so that the Eq. (1) is negligible, velocity v is then expressed by the following equation:

$$v = \frac{V_m'}{1 + \dfrac{k_4[s]}{k_5 + k_3}} \quad \cdots\cdots\cdots\cdots (7)$$

thus,

$$V_m' = \frac{k_3 k_5[\Sigma E]}{k_5 + k_3} \quad \cdots\cdots\cdots\cdots (8)$$

Therefore, K_m' obtained in the previous paper as the dissociation constant of ESS, should now be understood in the meaning of Eq. (9).

$$K_m' = (k_5 + k_3) / k_4 \quad \cdots\cdots\cdots\cdots (9)$$

V_m and V_m' in the Eq. (6) and Eq. (8) respectively, obtained in each temperature are in good agreement as given in Table IV.

TABLE IV

The Values of V_m and V_m' for Erythrocyte Cholinesterase at Different Temperatures

Temperature	V_m (CO_2)	V_m' (CO_2)
	cu.mm.	*cu.mm.*
15°	18.0	17.8
20°	21.7	20.8
25°	22.7	23.8
30°	25.9	26.8
37.5°	29.4	29.4

The experimental results can, therefore, be explained by the presumption $k_3 \ll k_5$ in Eq. (8). K_m' can thus be expressed as $K_m' = k_5/k_4$ in the meaning of the dissociation constant of Eq. (3).

As shown in Table V, K_m' obtained experimentally was of constant value and independent of the temperature.

From these relations, the

TABLE V

The Values of K_m' for Erythrocyte Cholinesterase at Different Temperatures

Temperature	K_m'
	mole/lit.
15°	1.1×10^{-2}
20°	1.1×10^{-2}
25°	1.4×10^{-2}
30°	1.1×10^{-2}
37.5°	1.2×10^{-2}

thermodynamic constants in the reaction of ESS complex formation were calculated as follows:

TABLE VI

The Thermodynamic Quantities of the Reaction of Eq.(3)

(310.5°K)

ΔF_3	-2.7 *kcal./mole*
ΔH_3	0 ,,
ΔS_3	$+8.8$ *cal./mole/deg.*

Entropy ΔS_3 was found here again in positive quantity.

SUMMARY

1. The temperature coefficient of erythrocyte cholinesterase was found as 1.3 and that of serum cholinesterase as 1.5.

2. The heat of activation of erythrocyte cholinesterase and serum cholinesterase were determined as 3.7 *kcal./mole* and 7.7 *kcal./mole* respectively.

3. The thermodynamic constants in the enzymatic reactions of cholinesterase in both types were calculated from the relationships between the Michaelis constant respectively the dissociation constant of ESS and the temperature.

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STUDIES ON THE METABOLISM OF ACID-SOLUBLE AND ACID-INSOLUBLE PHOSPHATE COMPOUNDS IN THE LIVER OF NORMAL AND THIAMINE-DEFICIENT RATS

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Researches during recent years have demonstrated the importance of phosphate compounds in the metabolism of animal tissues. For example, it was shown that adenosine-triphosphate (ATP) is a very important metabolite in the storage of utilizable energy of animal tissues (*1*), and nucleoprotein plays an important role as an energy transmitter between the reaction of catabolism and the synthesis of protein (*2*). On the other hand, it has also been demonstrated by many workers (*3–7*) that the utilization of molecular oxygen by animal tissues is coupled with phosphorylation reaction, and Limpmann (*1*) has emphasized the role of aerobic metabolism in the synthesis of high energy phosphate bond. As to the metabolism of phospholipid, there are some experimental indications that the formation of these compounds by phosphorylation depends on the tissue respiration as a source of energy. In this laboratory, Ogata, Hashimoto, and Enoki (*9*) (*10*) have recently demonstrated that the homogenate from the tissues of thiamine-deficient rats showed a significant decrease in the oxygen uptake and in the coupled phosphorylation as compared with the tissues of normal and pair-fed control rats, when α-ketoglutarate or pyruvate was used as the substrate. The fact that thiamine-deficient rats show much decrease in body weight as compared with pair-fed control rats (*11*), suggests that the synthesis or utilization of protein might be suppressed in those rats. The present paper deals with the experiment comparing the metabolic activity of the tissue phosphate compounds in thiamine-deficient rats with that in normal and pair-fed control rats. Some observation on the metabolic activity of phosphate compounds may be possible from the turnover studies using radioactive phosphorus (P^{32}).

EXPERIMENTAL

Treatment of animals—Rats weighing about 70 g. were divided into three groups, the first of which received a thiamine-free synthetic ration. The second, a control group, received a nutritionally complete synthetic ration *ad libitum*, and the third, pair-fed control group, was given the same ration as the first group but with thiamine added. All of the three groups received sufficient quantities of the components of the vitamin B_2 group (riboflavin, niacine, pyridoxine, pantothenate, and choline) for the complete nutrition. When remarkable deficiency-symptoms appeared in the thiamine-deficient group, all the rats of the three groups were used for the turnover experiment. Besides these experiments, the rats which recovered from thiamine-deficiency were tested as the third control. Some of the rats of the first group were administered 80 μg of thiamine daily for three days after bradycardia appeared in them, and then they were used for the turnover experiment.

For the turnover experiments, rats were fasted for 12 hours and injected with $Na_2HP^{32}O_4$ one hour before sacrifice. The dose of P^{32} was 15 microcurie (μC) per 100 g. of body weight, and this amount of $Na_2HP^{32}O_4$ was contained in 4 ml. of the carrier solution of 0.04 *M* Na_2HPO_4 (4.81 mg. of P). The injection was made intraperitoneally and the rats were killed by decapitation under methyl hexabital anaesthesia (5 mg. of cyclopan-soda were given per 100 g. of body weight) one hour after the injection of P^{32}. The abdomen was quickly opened and the liver was rapidly removed, weighed, and chilled in ice.

Analytical Method—The fractionation of acid soluble phosphate compounds was carried out principally by the procedure of Kaplan and Greenberg (*12*). As the labile P of ATP and ADP was regarded as the most important of all the acid soluble phosphate compounds, the fraction containing ATP and ADP was separated from the Ba-insoluble fraction as follows: The Ba-insoluble precipitate was dissolved in 1 ml. of 1 *N* acetic acid and 10 ml. of ice-cold water stirring with a glass rod. After standing for a few minutes, 1 ml. of 1 *N* HCl was added and the stirring was continued till a clear solution was obtained. This procedure was necessary to minimize the contamination of inorganic P^{32}. Two ml. of 20 per cent mercuric acetate were added to this solution and the mixture was kept in a refrigerator over night. The resulting precipitate, after being washed with dilute mercuric acetate solution, was suspended in 5 ml. of water, and was decomposed with hydrogen sulfite. One drop of 10 *N* H_2SO_4 was then added so as to remove any barium which might be present. The solution was then centrifuged, and the precipitate was washed with 2 ml. of water by centrifugation. The supernatant and washings were transferred to a volumetric flask, made up to 15 ml. with water, and filtered. The filtrate was aerated for one hour in a cold water bath. This fraction could be referred to as the ATP-ADP fraction, because it was proved to be free from fructose diphosphate by the Roe method (*13*) and contained only a negligible amount of inorganic phosphate. The labile P of ATP and ADP was determined by hydrolyzing an aliquot of this ATP-ADP fraction with 1 *N* HCl for 7 minutes at 100°. The supernatant of mercuric precipitation was treated with hydrogen sulfite and 10 *N* H_2SO_4 as in the case of ATP-ADP fraction, and precipitated by the addition of carrier phosphate and Mg-mixture described by Lehninger. The precipitate thereby formed was used for the determination of the specific activity of inorganic phosphate.

For the fractionation of the acid-insoluble phosphate compounds, 1 g. of the liver was homogenized in a Potter-Elvehjem glass homogenizer with ice cold water. One ml. of the homogenate was then fractionated by the following method which was a slight modification of the Schmidt-Thannhauser method (*14*) and of the Schneider method (*15*). To the homogenate 2.5 ml. of 10 per cent trichloroacetic acid (TCA) were added, and the mixture was allowed to stand for at least 10 minutes in ice. The precipitated material was centrifuged down and washed with 2.5 ml. of ice-cold 10 per cent TCA. The precipitate was extracted three times with ice-cold 95 per cent ethanol, and each extract was combined to provide the lipid fraction (lipid-P). The residue was treated with 1 ml. of 1 *N* KOH and was incubated at 37° over night. The alkaline digest was then treated with 0.2 ml. of 6 *N* HCl and 1 ml. of 5 per cent TCA (both cold) and was placed in an ice cold bath for 10 minutes. The resulting precipitate (A) was centrifuged down and washed twice with 2 ml. each of cold 5 per cent TCA. The supernatant solution and washings were mixed with 0.2 ml. of 0.025 *M* Na_2HPO_4 and 1.0 ml. of Mg-mixture (0.1 *M* $MgSO_4$-1 *M* NH_4Cl), and made up to 10 ml. with 0.8 *N* NH_4OH. After standing for at least 2 hours in a refrigerator, the precipitate formed was filtered off and the filtrate was regarded as the ribonucleic acid fraction (RNA–P). The washed precipitate (A) was digested with 5.0 ml. of 5 per cent TCA for 15 minutes in a boiling water bath and centrifuged. This procedure was repeated again, the two TCA extracts were combined, and set aside as the desoxyribonucleic acid fraction (DNA–P).

A suitable portion of each of the above fractions was taken for the determination of P by the method of Fiske-Subbarow (*16*).

The Measurement of Radioactivity—For the measurement of the radioactivity of the acid-soluble organic compounds, the method of Lehninger (*17*) was employed, i.e., the contamination of inorganic P^{32} was precipitated by the addition of carrier phosphate and Mg-mixture described by Lehninger (17). The precipitation of inorganic P as $MgNH_4PO_4$ was proved to accompany the adsorption of ATP and ADP by experiments by Lehninger, and this fact was also acknowledged by us by the preliminary experiment. Accordingly, in the present procedure, only a very small amount of carrier P, i.e., 0.05 ml. of 0.025 *M* phosphate, which is one-fourth of the amount described by Lehninger, was used to precipitate the inorganic phosphate contained in ATP-ADP fraction. This quantity of the carrier phosphate was proved to be satisfactory to precipitate the contaminated inorganic phosphate of this fraction by the preliminary experiment in which the inorganic P^{32} was added directly to the liver homogenate of a rat which was not injected with P^{32}.

For the measurement of the radioactivity of the acid-insoluble fractions, portions of each solution were digested with 10 *N* H_2SO_4 in an oven at 130–160° for about 2 hours, and after they were cooled moderately, 1–2 drops each of 30 per cent H_2O_2 and concentrated HNO_3 were added to them. They were again replaced in the oven for about 2 hours, cooled moderately, a small amount of water was added to the residues, and they were heated at 100° for 10 minutes. Each solution digested in this manner was mixed with 5 ml. of 0.04 *M* Na_2HPO_4 and 5 ml. of Mg-mixture (55 g. of $MgCl_2 \cdot 6H_2O$ and 100 g. of NH_4Cl in a liter of 1.5 *M* NH_4OH) and was made alkaline with concentrated NH_4OH. After standing over night in a refrigerator, the resulting precipitate was collected on a glass filter, and dried completely by infra-red rays. The radioactivity of this dry precipitate was measured.

Besides the experiment mentioned above, about 0.05 μC of P^{32} was added in the form of $Na_2HP^{32}O_4$ to the initial liver homogenate, which was obtained from the rat not injected with P^{32}, and the radioactivity of each fraction was measured. By this experiment it was proved that each of the above fractions was not contaminated with inorganic P^{32}.

P^{32} is expressed in terms of relative specific activity (R.S.A.) according both to Hevesy (*18*), who expressed the specific activity of each fraction as per cent of the specific activity of the inorganic fraction, and to Sacks (*19*), who expressed R.S.A. as the value of specific activity multiplied by body weight.

RESULTS

Acid-soluble Phosphate Compounds—Following fractions were analyzed as the acid-soluble phosphate compounds: inorganic orthophosphate, the labile P of ATP-ADP, Hg precipitate, and alcohol fraction. According to Kaplan and Greenberg (*12*) the Hg-fraction contains nucleotide other than ATP and ADP, and the alcohol fraction contains glycerophosphate, glucose-1-phosphate and glucose-6-phosphate. The results are shown in Tables I and II.

Table I indicates the distribution of P^{31} of the acid-soluble fractions. By these experiments it was clarified that the distribution of the acid-soluble phosphate compounds of liver does not change in the case of thiamine-deficient rats as compared with normal and pair-fed control. However, as shown in Table II, there was a significant decrease in the R.S.A. (Hevesy and Sacks) of labile P of ATP-ADP of thiamine-deficient rats as compared with normal and pair-fed control, and in the case of recovered rats the R.S.A. was restored to the range of normal rats. With respect to the R.S.A. (Hevesy and Sacks) of Hg-fraction and the R.S.A. (Sacks) of inorganic phosphate of liver and blood plasma, no significant difference was found between thiamine-deficient, pair-fed control, normal, and recovered rats. Thiamine-deficient and recovered rats showed some decrease in the R.S.A. of the alcohol fraction.

Acid-insoluble Phosphate Compounds—Following fractions were analyzed as the acid-insoluble phosphate compounds: lipid, RNA, and DNA.

TABLE I

Distribution of Acid-Suluble Phosphate fractionated from Livers of Normal, Pair-fed Control, Thiamine-deficient, and Recovered Rats

(Values as mg. P per 100 g. wet weight of liver)

	Number of rats	Total acid-soluble P	Ba-insoluble fraction			Ba-soluble fraction	
			Total Ba-insoluble P	Inorganic P	Labile P of ATP, ADP	Hg fraction	Alcohol fraction
Normal rats	11	104.3±1.1†	57.1±1.4	29.1±1.0	10.2±0.4	18.2±1.3	18.4±0.6
Pair-fed control rats	7	99.3±1.4	59.5±1.3	30.6±1.1	10.3±0.7	13.5±0.7	17.4±0.6
Thiamine-deficient rats	9	100.1±1.8	57.7±2.1	31.6±1.1	10.2±0.4	12.8±1.1	17.7±0.4
Recovered rats	3	93.8±1.0	51.7±0.8	30.4±0.9	9.5±0.2	15.4±0.5	17.8±0.4

† The measure of variability is the probable error of the mean.

The concentration of P^{31} in the lipid fraction of thiamine-deficient rats decreased remarkably, but those in ribonucleic and desoxyribonucleic acids showed no significant change. The R.S.A. (Sacks) of each of the lipid-, RNA- and DNA-P of thiamine-deficient rats showed significant decrease. The R.S.A. (Sacks) of each of the above fractions of pair-fed control rats was between the values of normal and thiamine-deficient rats. In the recovered animals, the above decreased values of R.S.A. of thiamine-deficient rats were restored to some extent.

DISCUSSION

There are some difficulties in the quantitative determination of the rate of the renewal of phosphorus in tissue compounds. Especially, in our case, the body weight of thiamine-deficient and pair-fed control rats was much decreased as compared with control rats, and thus it was necessary for us to correct the values obtained in respect to this factor. For acid-soluble phosphate compounds the R.S.A. proposed by Hevesy (*18*) is considered to be very reasonable, as the phosphorus of these compounds may be regarded as being derived directly from the inorganic phosphate in the liver cell, and the R.S.A. values obtained are independent of the differences of the absorption of inorganic phosphate

TABLE II

The Relative Specific Activity of each Acid-soluble Fraction of Liver of Normal, Pair-fed Control, Thiamine-deficient, and Recovered Rats

	Number of rats	Relative specific activity* (Hevesy)			Relative specific activity (Sacks)				
		Labile P of ATP, ADP	Hg fraction	Alcohol fraction	Liver inorganic P	Labile P of ATP, ADP	Hg fraction	Alcohol fraction	Plasma inorganic P
Normal rats	5	79.3±3.0†	23.3±1.4	46.8±4.1	4130±278	3363±238	987±65	2159±120	4354±337
Pair-fed control rats	3	81.5±3.9	22.3±1.2	42.3±4.8	3705±340	3002±144	808±42	1374±342	4428±330
Thiamine-deficient rats	5	56.0±1.9	21.6±3.2	38.2±2.1	4390±296	2097±157	868±40	1490±145	4000±480
Recovered rats	3	79.0±1.6	20.0±0.7	28.0±1.8	4310± 56	3423± 75	817±66	1160± 72	4720±450

† The measure of variability is the probable error of the mean.
* The specific activity of each fraction as % of the specific activity of inorganic fraction.

TABLE III

Distribution of P^{31} and P^{32}, and Relative Specific Activity (Sacks) of each Acid-insoluble Fraction of Liver of Normal, Pair-fed Control, Thiamine-deficient, and Recovered Rats

	Number of rats	P^{31} (mg./100 g. liver)			P^{32}*(Count/min./100g. liver)			Relative specific activity (Sacks)		
		Lipid-P	Nucleic acid-P		Lipid-P	Nucleic acid-P		Lipid-P	Nucleic acid-P	
			RNA	DNA		RNA	DNA		RNA	DNA
Normal rats	4	133.2±1.88†	94.6±1.79	25.4±1.29	244±15.6	66±3.2	13±2.0	245±26.0	82±5.3	61±7.2
Pair-fed control rats	3	119.4±7.59	89.8±1.85	26.3±3.49	339±12.0	92±8.0	19±1.9	231±19.9	82±6.8	60±3.4
Thiamine-deficient rats	5	102.9±3.75	85.5±1.60	30.8±0.49	243±46.7	62±7.6	15±2.2	174±30.8	54±6.6	36±3.1
Recovered rats	3	127.7±1.42	92.1±0.38	24.4±0.62	228±17.3	57±4.8	12±1.7	191± 5.0	63±4.4	52±6.8

* Calculated from the counts of the injected P^{22} as 1000. † The measure of variability is the probable error of the mean.

through the cell membrane at various pathological states of animals. However, as stated by Sacks (*19, 20*) there is a possibility that phosphate enters the liver cell by the formation of ATP, ADP, or glucose-1-phosphate. Therefore, the calculation of R.S.A. proposed by Sacks was also adopted in the present experiment in addition to the calculation proposed by Hevesy. According to Sacks (*19*) this R.S.A. serves to make the results obtained with different injection doses and animals of different body weights quantitatively comparable. The present result shows that the rate of the renewal of the labile P of ATP and ADP is very large in the liver of normal and pair-fed control rats. The specific activity of this farction is about 80 per cent of that of inorganic phosphate one hour after the intraperitoneal injection of P^{32} in these animals. This value is much higher than that reported by Kaplan and Greenberg (*21*) and is almost the same as that of Sacks (*19*). The R.S.A. (Hevesy and Sacks) of labile P of ATP and ADP in the liver of thiamine-deficient rats decreases below that of control animals, and this decrease is restored in the recovered animals. In our former reports (*9, 10*) the activity of the oxidation and the coupled phosphorylation of the tissues homogenates of thiamine-deficient rats decreased when α-ketoglutarate or pyruvate was added as oxidizable substrate, and the addition of catalytic amounts of diphosphothiamine restored this activity. The decrease of R.S.A. of the labile P of ATP and ADP of liver may be explained by these former experimental results. The decrease of the turnover rate of phosphorus of the phospholipid fraction of thiamine-deficient rats indicates that the formation of this compound by phosphorylation is dependent on respiration as a source of energy (*8*). The formation of the two phosphate ester bonds in the phospholipid molecule requires a free energy increase of 5000 cal. (*28*), and the oxidative processes are required to supply this energy need by coupling with the reaction which produces energy-rich compounds analogous to, if not identical with, ATP, acetylphosphate and others. The renewal of phosphorus in nucleic acid has been studied by many workers (*23–27*) in recent years, but there are only a few reports concerning the incorporation of P^{32} into the nucleic acid in the very early stage after the injection of inorganic P^{32}-compounds. In the present study it was proved that the incorporation of P^{32} into both RNA and DNA in liver occurs one hour after the intraperitoneal injection of $Na_2HP^{32}O_4$. From the study of the renewal of P^{32} in the phosphate compounds of yeast cells, Spiegelman and Kamen(*2*)suggested that nucleic acids were critically important in controlling protein synthesis. The thiamine-deficient rats show a remarkable decrease in body weight as compared with pair-fed control, and this fact suggests the presence of some metabolic defect in utilizing protein in thiamine-deficient tissues. There may be some relationship between the decrease of the rate of the renewal of phosphate in nucleic acids and the metabolic defect in protein utilization in thiamine-deficient tissues.

SUMMARY

The phosphates of acid-soluble and acid-insoluble fractions of rat liver were fractionated one hour after the intraperitoneal injection of $Na_2HP^{32}O_4$, and the renewal rate of phosphorus in thiamine-deficient rats was compared with normal and pair-fed rats. For the fractionation of acid-soluble phosphate compounds, the method of Kaplan and Greenberg was principally employed. The ATP-ADP fraction was separated from the Ba-insoluble fraction, and the contaminated inorganic P^{32} was precipitated by the addition of a very small amount of carrier phosphate. For the fractionation of acid-insoluble phosphate compounds Schmidt-Thannhauser's and Schneider's methods were used with a slight modification. The following fractions were separated and analyzed: (A) Acid-soluble fractions: i) labile P of ATP-ADP fraction, ii) inorganic phosphate from Ba-insoluble precipitate, iii) Hg fraction, and iv) alcohol fraction from Ba-soluble fraction; (B) Acid-insoluble fractions: i) lipid fraction, ii) RNA, iii) DNA. The results were as follows:

1. No remarkable difference was observed in the distribution of P^{31} in all the fractions described above, except the lipid fraction, between normal, pair-fed, and thiamine-deficient rats.

2. The thiamine-deficient rats showed significant decrease in the turnover rate of the labile P of ATP and ADP compared with normal and pair-fed control rats, and this decrease was restored to the normal range in the recovered rats. Such remarkable changes of the rate of the renewal was not found in the other acid-soluble fractions; only some decrease was observed in the alcohol-fraction of thiamine-deficient and restored rats.

3. The rate of the renewal of lipid, RNA, and DNA phosphate in thiamine-deficient rats showed significant decrease and that of pair-fed control rats was found to be between the values of normal and thiamine-deficient rats. In the recovered animals these decreased values of thiamine-deficient rats were restored to some extent.

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FREE AND ESTERIFIED VITAMIN A IN FISH OILS; CHROMATOGRAPHIC SEPARATION AND COLORIMETRIC DETERMINATION*

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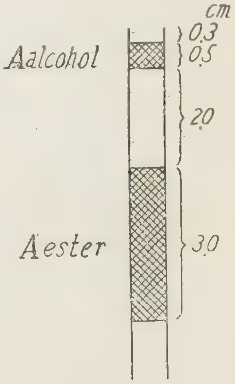
Müller (*1*) reported in a series of articles the condition of activation and deactivation of alumina and using the alumina thus prepared he showed the possibility of separating vitamin A from its esters. Eden (*2*) reported that by allowing to flow the vitamin A solution in petroleum benzine (PB) through the column of alumina and by subsequent development with PB the vitamin A alcohol is adorbed on alumina, while the vitamin A ester is eluted and flows through the column completely. But the fractional separation of vitamin A is found not always to be attainable, depending largely upon the nature of alumina.

We tried the chromatography with the activated alumina "BL6," which was found by us (Fujita and Aoyama (*3*)) to be adequate for separating vitamin D from vitamin A. After developing with 10 ml. of petroleum ether (PE), we found that both fractions of vitamin A are not separated but remain adsorbed on the upper part of the column. By using the alumina of Merck, Germany both fractions were separated but there appeared another zone between the two zones, which became light violet after adding several drops of $SbCl_3$ reagent on the section of the column. In order to weaken the activity of alumina we washed the alumina "BL6" with water, and one half was dried at 150° for one hour, while the other half was dried at 45° overnight. When the former was used as adsorbent, neither fraction of vitamin A separated even after developing with 10 ml. of the solvent.



PB 3 ml.

FIG. 1. Fractionation of free and esterified vitamin A.

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* The results of this paper were presented before the Research Committe for Fat-soluble Vitamins on September 26, 1950.

On the contrary, when the latter was used, the separation was complete with 3 ml. of the solvent as is shown in Fig. 1. Between both fractions there appeared a zone of about 2 cm. in width, which remain colorless after after adding $SbCl_3$ reagent. Thus the separation of both fractions of vitamin A become easily attainable.

PRINCIPLE

The mixture of vitamin A alcohol and ester is allowed to flow through the column of the weakly activated alumina and is developed with PB alone. The ester flows readily through the column, while the free alcohol remains adsorbed on the upper part of the column. By elution with PB containing acetone the free alcohol flows out of the tube and the non-A substances, which give color reaction with $SbCl_3$ reagent remain adsorbed on the column. Each fraction, which is separately collected in the test-tube is subjected to our colorimetric determination with glycerol dichlorohydrin (Fujita and Aoyama (*4*)). By this method, each fraction of vitamin A gives the same value whether it is saponified before the estimation or not.

REAGENTS

1. Weakly activated alumina.

The activated alumina " BL6 " of the Nippon Alumina Co., Setagaya Tokyo (average size of particles 10–20 μ, pH 6.8) is washed with water, spread on the plate with about 5 mm. in thickness and is dried at 40° for 9.5–10.3 hours with occasional mixing.

The residual water content, obtained as the difference of drying at 40° and to drying at 150° to the constancy of weight was found to be 0.167 g. to one g. of the anhydrous alumina.

Using the alumina, thus completely dried, the adequate amount of water to be added was found to be 0.125–0.165 ml. to each g. of dried preparation.

The activated alumina " KK1 " of the Scientific Research Institute, Komagome-Kamifujimae, Bunkyo-ku, Tokyo (average size of particles 2–10 μ, pH 6.8) can be similarly used instead of " BL6 " after the following treatment.

" KK1 " is washed with water and is dried at room temperature about 48 hours. One ml. of water is added to 40 g. of this dried alumina and mixed well.

The residual water content of the alumina thus treated was found to be 0.160 g. to one g. of the anhydrous material. Using the alumina sufficiently dried at 150° as above, the adequate amount of water to be added to one g. of anhydrous material was found to be 0.135–0.160 ml. When " KK1 " thus treated is used as adsorption column and 1 ml. of the PB solution containing 11.3 μg. of vitamin A alcohol and 11.7 μg. of vitamin A ester separated from liver oil is added to the column and is developed twice with 2 ml. of PB, the alcohol fraction appears from 0.6 cm. below the surface of the column (about 1 cm. in width). The ester raction appear about 5 cm. below the lower limit of the alcohol fraction.

2. Petroleum benzine.
3. 20% (v/v) acetone in PB.

All solvents must be free from peroxide. Treatment for removing peroxide is described in the previous paper (Fujita and Aoyama (*5*)).

PROCEDURE

About 10 mg. of liver oil (containing less than 10 μg. of vitamin A alcohol and 20 μg. of A ester) are dissolved in 5 ml. of PB. One ml. of the solution is subjected to chromatography on weakly activated alumina. Adsorption column is about 2 cm. in lenght and 0.7 cm. in width. The procedure of chromatography is the same as is described in the following paper concerning the separation of vitamin A from non-vitamin A materials (Fujita and Aoyama (*5*)).

After flow of 2 ml. of PB twice, vitamin A ester comes completely out of the tube, which is collected in a test-btue. In order to ascertain that the vitamin A ester has come out completely, further 1 ml. of PB is allowed to flow through the column and the eluate is tested fluorometrically by ultraviolet irradiation or colorimetrically by adding $SbCl_3$ reagent. If the vitamin reaction is negative, it shows that the removal of vitamin A ester is complete. Then the column is rinsed twice with 2 ml. of 20 per cent aceton in PB, whereby the vitaimin A alcohol comes out completely, which is collected in a test-tube. The complete elution of the vitamin A alcohol is ascertained similarly by allowing to flow 1 ml. more of the solvent through the column and by examining the eluate to be free from vitamin A.

The time necessary for chromatography is about 5–6 minutes. Under this condition both forms of vitamin A come out of the tube completely and the previously added vitamin is practically completely recovered.

Remarks.

1. The values for vitamin A ester are expressed as vitamin A alcohol.
2. The vitamin A ester fraction contains besides A ester more or less amount of non-A substances, but the amount is in case of most liver oils usually negligible (5).

Example.

Liver oil of *Theragra chalcogrmma.*

Direct estimation without fractional separation:

$$0.880\times 30\times 5\times 100=13{,}200\ (\mu g/gm.)$$

$$\text{Vitamin A alcohol:}\quad 0.017\times 30\times 5\times 100=255\ (\mu g./gm.)$$

$$\text{Vitamin A ester:}\quad 0.855\times 30\times 5\times 100=12{,}800\ (\mu g./gm.)$$

$$\text{Total:}\quad 13{,}100\ (\mu g./gm.)$$

$$\frac{\text{Alcohol}}{\text{Total}}=\frac{0.017}{0.880}\times 100=1.9\ \%$$

EXPERIMENTAL

Recovery Test—10 ml. of PB are added to 10 mg. of the liver oil of *Theragra chalcogramma* and 1 ml. of the solution is used as main test. 3.96 μg. of the vitamin A ester obtained by chromatographic separation of liver oil were used for recovery test of vitamin A ester and 3.3 μg. of the vitamin A alcohol obtained by chromatographic separation of the unsaponifiable matter of liver oil were used for recovery test of vitamin A alcohol. As the results of the experiment the recovery was 98 per cent for ester and 100 per cent for alcohol.

Distribution of Free and Esterified Form of Vitamin A in Liver Oils—The results obtained with several liver oils are shown in Table I and it is clear from the table that more than 97 per cent of vitamin A in liver oil exist in esterified form and only 2–3 per cent in free form. This finding confirms the report by Kascher and Baxter (*6*) rather than the report by Sobotka (*7*).

TABLE I

Distribution of Free and Esterified Vitamin A in Liver Oils

Liver oil of	Vitamin A content (*μg./gm.*)			Free/Total
	Free	Ester	Total	
				per cent
Squatina japonica	75	4,080	4,155	1.8
Katsuwonus vagans	75	2,050	2,125	3.5
Seriola quinqueradiata	90	3,540	3,630	2,5
Squalus sackleyi	105	3,880	3,985	2.6
Balaenoptera physalus	75	3,210	3,285	2.3

Conditions for Activating Alumina—Conditions of activation are important for fractinating free and esterified vitamin A. Heating at 25° for 20 hours is not enough for adsorbing vitamin A alcohol. After flow of only 1 ml. of PB the upper limit of the vitamin A alcohol zone comes down already to 1.5 cm. from the upper surface of the column. After heating at 45° for 15 hours the separation of both forms of vitamin A was not complete, the esterified form remaining about 0.8 cm. below the upper surface of the column after flow of 4 ml. of PB. After heating at 60° for one hour the adsorption of the free form is incomplete, the free form coming down to 3 cm. below the surface after flow of 2 ml. of PB. After heating at 60° for 2 hours the adsorption was almost complete. After heating at 100° for one hour neither form was separated. Since, after heating at 60°, the time required for suitable adsorption was somewhat close to the time for unsuitable adsorption, the heating was tested at 40° for various hours. The results are summarized in Table II. The most adequate duration of heating was found to be 9.5–10.5

TABLE II

Effects of Heating on the Activation of Alumina

(A alcohol: 10 μg. A ester: 20 μg. PB: 4 ml.)

a: Distance from the upper surface to the lower limit of the vitamin A alcohol zone. (cm.)

b: Distance from the upper surface to the upper limit of the vitamin A ester zone (cm.).

c: Width of the vitamin A ester zone (cm.).

+: Suitable, ±: Almost suitable, —: Unsuitable.

Alumina	Heated at	Heated for	a	b	c	Judgement
		hours	*cm.*	*cm.*	*cm.*	
BL6	40°	8	not separated			—
		9	1.8	5	3	±
		9.5	1.5	4	4	+
		10	1.2	3.5	4	+
		10.5	1.0	3	4	+
		11	0.8	2.2	3.5	±
		11.5	0.8	1.5	3.2	—

	Heated at	1 ml. of water is added to dried alumina	a	b	c	Judgement
KK1	Room temperature for 24 hours	$0^{g.}$	not separated			—
		20	3	10	almost out	+
		40	1.6	6.6	partially out	+
		80	1.0	3	3	±

hours, whereby the intermediary zone between both zones of vitamin A was about 2–2.5 cm. in width. Thus the separation of both forms was then complete. Incidentally, when 0.5 ml. of lycopene solution with E (S_{47}; 0.5 cm.) 1.0 is adsorbed on the alumina thus treated and is developed with 4 ml. of PB, the upper limit of the lycopene zone lied about 4.5–6 cm. below the surface of the column.

SUMMARY

1. An adequate method for separating vitamin A alcohol from vitamin A ester is described in detail.

2. For this purpose the appropriate activation of alumina is necessary, the condition of which was described.

3. Usually more than 97 per cent of the vitamin A in liver oil is in the esterfied form.

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COLORIMETRIC AND FLUOROMETRIC DETERMINATION OF VITAMIN A IN FISH LIVER OILS; CHROMATOGRAPHIC SEPARATION OF VITAMIN A AND NON-VITAMIN A MATERIALS IN THE UNSAPONIFIABLE MATTER*

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The assay methods for vitamin A, which are now commonly used, namely antimony trichloride method (Carr and Price (*1*)), glycerol dichlorohydrin method (Sobel and Werbin (*2*)) or estimating the absorption coefficient at 328 mμ give vitamin A value in case of liver oils and other biological materials, which are not the actual vitamin A values but are more or less interfered by non-vitamin A reacting substances.

For the estimation of the actual vitamin A values, corrections are introduced by Morton and Stubbs (*3*) and Oser (*4*), as described in the U.S. Pharmacopeia XIV (1950), on the assumption that the extinction curve of non-A substances between 325 and 334 mμ runs linearly. However, this latter assumption is not always true as shown in the following experiments. Gridgeman, Savage and Gibson (*5*) and Barua and Morton (*6*) have separated vitamin A and non-vitamin A of whale-liver oils by chromatography on alumina. Being inaccessible to these papers, we have independently engaged in working out a satisfactory method for separating the actual vitamin A from the intermingled non-A interfering substances by chromatography on activated alumina and we believe to have succeeded in easily and exactly estimating the actual vitamin values.

For estimating the actual vitamin A we have found two methods, namely the direct and the indirect. By applying to the fish liver oils both methods give the same values but the former is more preferable for the practical use.

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* The results of this paper were presented before the Research Committee for Fat-soluble Vitamins on September 26, 1950.

I. THE INDIRECT ESTIMATION OF THE ACTUAL VITAMIN A.

Principle—After saponification of liver oil, the unsaponifiable matter (USM) is chromatographically fractionated into vitamin A alcohol and non-A fractions. The latter fractions consist of several substances, which are estimated in toto. The actual vitamin A value is obtained by subtracting the non-A from the total.

Reagents—

1. 5 per cent KOH in ethyl alcohol (EtOH).
2. 5 per cent KOH in 60% (v/v) methanol.
3. Benzene.
4. Petroleum benzine (PB).
5. Acetone.
6. 10 per cent (v./v.) acetone in PB.

All solvents must be free from peroxide. When a small amount of ammonium ferrous sulfate and KOH and water are mixed and then potassium thiocyanate is added, the solvent must not become red. To remove peroxide about 1 gm. of ammonium ferrous sulfate and a small piece of KOH and 2 ml. of water are added to 10 ml. of solvent, thoroughly mixed and let it stand for 1.5 hours with occasional shaking. The amount of ferrous salt to be added is dependent on the amount of existing peroxide. The supernatant liquid is distilled after dehydration and asceratained with thiocyanate for the absence of peroxide.

For the fluorometric estimation each solvent must be free from fluorescence. For removal of fluorescence the solvent must be redistilled. For GDH method the complete removal of fluorescence is not necessary.

7. Activated alumina. The alumina " BL6 " of Nippon Alumina Co., Setagaya, Tokyo (average size of the particles was 10–20 μ, pH 6.8) can be used for the present purpose. For fluorometric estimation the alumina is rinsed with methanol and PB which are free from fluorescence and is heated at 400–500° for one hour.

The residual water content of alumina was determined as follows. An aliquot of the sample was heated at 450° to the constancy of weight. The water content was found to be 0.0038 g. per one g. of anhydrons material. The addition of 0–0.005 g. water to one g. of anhydrous material was found to be equally adequate for this purpose. The heating of the sample at 450° for 3 hours was proved to be equally suitable as that for one hour. The preparation dried at 150° to the constancy of weight was not active enough for this purpose.)

The " KK1 " of the Scientific Research Institute, Komagome, Bunkyo-ku, Tokyo (average size of the paticles was 2–10 μ, pH 6.8) can also be used after washing with water, drying at room temperature for 48 hours and heating at 150° for one hour.

The residual water content of the alumina was determined after heating at 150° to the constancy of weight. It was found to contain 0.00018 g. water per one g. of anhydrous material. The addition of 0–0.0002 g. water to one g. of anhydrous material was found to be equally adequate for the purpose.

8. Standard solution of Vitamin A. As pure vitamin A preparation was unavailable to us for the standard solution, we adopted the following procedure. Liver oil was saponified, the USM was chromatographically separated, the vitamin A alcohol zone of the chromatogram was eluted, rinsed with water as described in the procedure; the extinction curve of this sample agreed quite well with that of the pure vitamin A alcohol as is shown in Fig. 4–6 (See also experimental parts). Absorption maxima lie at 325 mμ in alcohol and at 331 mμ in benzene. The benzene solution is stored in a refrigerator under CO_2 gas. It is preparable to prepare the solution freshly every day or at least once a week. The vitamin A content of the solution is estimated by GDH method Fujita and Aoyama (*4*)) every day before the estimation and by diluting with benzene the vitamin A solution containing exactly 1 μg./ml. which is used as a standard solution is prepared. It is stored under CO_2.

Precedure—A suitable amount of liver oil (usually about 10 mg.) is placed in a centrifuge-tube with stopper (about 50 ml. capacity) and 5 ml. of 5% KOH-EtOH are added. The solution is bubbled with hydrogen gas and heated in water-bath at 75° for 30 minutes. It is cooled with running water and 10 ml. of benzene are added, the solution being bubbled with H_2. Then further 10 ml. of water are added and it is bubbled with H_2. After centrifugation the benzene layer is transferred to another centrifuge-tube with stopper (about 50 ml. capacity), rinsed with 20 ml. of 5 per cent KOH in 60 per cent methanol and rinsed with water 2–3 times. The extract is usually clear. If turbid, the washing with water is repeated, the gas space being always filled with H_2 or CO_2. The benzene layer is made with benzene to exactly 10.0 ml. (Solution B).

1) *Estimation of Total Value:* Solution B is diluted with benzene to 1:10 and 1 ml. of the diluted solution is made with benzene to 5 ml. and is determined flourometrically (Titration with standard A solution containing 1 μg./ml. of vitamin A as was described by us (Fujita and Aoyama (*3*)). From this value the total fluorescence value expressed as vitamin A in 1 g. of liver oil is calculated (*m*).

2) *Estimation of Non-A Substances:* 5 ml. of solution B are taken in a test-tube, benzene is evaporated in vacuum and the residue is dissolved in 2 ml. of PB. It is subjected to chromatography on activated alumina. The size of the adsorption column is 10 × 0.72 cm., using 2.9 g. of alumina " BL6 " or 2.5 g. of " KKl." The rate of flow was 40–50 drops per minute for " BL6 " or 35–45 drops per minute for " KKl." For development 50 ml. of 10 per cent acetone in PB were used. Usually 5 zones appeared after development at ultraviolet irradiation or after addition of $SbCl_3$ reagent on the section of the column (Fig. 1). The

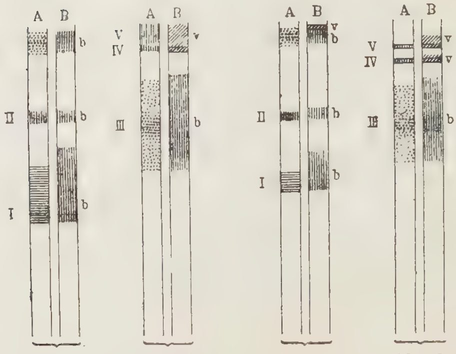

FIG. 1. Fractionation of liver oil components.
I. Liver oil of *Theragra chalcogrmma.*
II. Liver oil of *Squalus sackleyi.*
A. Color of fluorescence.
B. Color of Carr-Price reaction.

I zone comes out of the tube after 7 ml. of the developing solvent having passed through. The II zone comes out of the tube after passage of about 50 ml. of the solvent. The III zone is vitamin A alcohol, which is about 5 cm. in width and fluoresces greenish-yellow at ultraviolet irradiation. Above the III zone lies a somewhat sharp IV zone and a relatively weak V zone. The adsorption column is pushed out of the tube, all the zones except III are collected, and thoroughly extracted with 5 ml. of PB and 5 ml. of methanol. The extract is transferred to another test-tube and mixed with 5 ml. of water, whereby the PB layer is separated. The same extraction is repeated once more and the collected PB layer is made to 100 ml. 5 ml. of the solution are evaporated, dissolved in 5 ml. of benzene and is estimated fluorometrically. The non-A fluorescent substances expressed as vitamin A alcohol in 1 g. of liver oil is calculated (m_0). Then the actual vitamin A is $m-m_0$ (μg./g.).

When 80 μg. of vitamin A is added to the liver oil, and the whole is treated as described above, the added amount is practically completely recovered.

Remarks.

(1) Glycerol dichlorohydrin method can equally be used for estimating vitamin A (*cf.*, Fujita and Aoyama (*6*)). The values agree practically completely with those determined fluorometrically.

(2) In the above procedure the vitamin A alsohol can not be estimated directly, as it is not stable enough in the course of chromatography, which takes about 2 hours.

On the contrary, the non-A substances are quite stable during the whole treatment. Therefore, the actual vitamin value is obtained by subtracting the non-A value from the total.

II. THE DIRECT ESTIMATION OF ACTUAL VITAMIN A.

Principle—By using the weakly activated alumina the vitamin A alcohol can be collected in several minutes. Under this condition the vitamin A is stable enough and the added amount is practically completely recovered. In this procedure the separation of vitamin A and D is not complete. But the participation of vitamin D is most cases negligible, especially in fluorometric estimation and the values of direct method usually agree quite well with those of indirect method. In case of whale liver oil the vitamin A alcohol and kitol can be separately estimated.

Reagents.

(1) Weakly activated alumina.

The alumina " BL6 " of Nippon Alumina Co. Setagaya, Tokyo (average size of particles 10–20 μ, pH 6.8) is washed with water and is spread in a dish to the thickness of 5 mm. and is dried at 40° for 9.5–10.5 hours with occasional mixing. The residual water content was determined by drying at 150° to the constancy of weight. It was found to contain 0.167 g. water per 1 g. of the anhydrous sample. The addition of 0.125–0.165 ml. water to each 1 g. of the dried sample was found to be equally adequate for the purpose.

The alumina " KK1 " of the Scientific Research Institute, Komagome, Tokyo (average size of particles 2–10 μ, pH 6.8) can also be used after following treatment. The alumina is washed with water, spread in a dish to the thickness of 5 mm. and dried at the room temperature for 48 hours, 1.0 ml. of water is added to 40 g. of the alumina treated as above and is thoroughly mixed. The residual water content of the alumina thus treated was found to be 0.160 g. per 1 g. of the anhydrous material. Using the sample, dried at 150° to the constancy of weight, the adequate amount of water to be added to it was found to be 0.135–0.160 ml. per 1 g.

(2) Other reagents are the same as were described in the indirect method.

Reagents.

Procedure—About 10 mg. of liver oil containing less than 10 μg. of vitamin A are saponified and the NSM is extracted with benzene as described above.

The benzene layer is evaporated in vacuum and the residue is dissolved in 2 ml. of PB. One ml. of the solution is subjected to chromatography. Adsorption tube is about 0.72 cm. in diameter. The column is about 2.5 cm. in length. The amount of alumina for one column was about 0.8 g. The solution is transferred to the column. The rate of flow is 60–70 drops per minute for " BL6 " and 45–55 drops for " KK1." The development is performed with 2 ml. of PB two times, through which the **I** zone comes out rapidly and then the **II** zone follows. In order to ascertain that vitamin A alcohol does not come out, further 1 ml. of PB is added and the filtrate is examined for vitamin A by fluorescence or color reaction with $SbCl_3$ reagent. If the procedure is carried out rightly, the filtrate must be free from vitamin A alcohol.

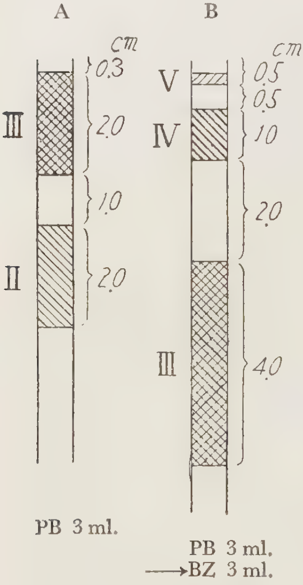

Fig. 2. Chromatograph of liver oil.

In order to show the relationship between the **II** zone and vitamin A zone (**III**), the chromatographic development using the column of 9 cm. in length is shown in Fig. 2 A. The zones can be observed in the same position, either by fluorescence under ultraviolet irradition or by color reaction using $SbCl_3$ reagent.

Then the column is rinsed with 2 ml. of benzene two times and the filtrates are collected, which contain the total vitamin A alcohol. It is estimated colorimetrically or fluorometrically. The whole development takes about 3–4 minutes. In order to ascertain that the vitamin A alcohol has completely come out, further 1 ml. of benzene is added and the filtrate is examined. It must be free from fluorescence. The chromatographic relationship can be seen from Fig. 2B, whereby the adsorption column of 9 cm. in length was used. Above the vitamin A alcohol zone (**III**) there are **IV** and **V** zones. The **IV** zone is blue like **III** after adding $SbCl_3$ reagent, but the **V** zone is violet. The fluorescence of **IV** is also the same as **III** but **V** is somewhat bluish fluorescent.

EXPERIMENTAL

1. *Chromatographic Separation of Vitamin A Alcohol and Non-A Fractions*—10 mg. of liver oil of *Theragra chalcogramma* is saponified and the NSM is subjected to chromatography on alumina " BL6." It is

developed with 4 and 40 ml. of 10 per cent acetone in PB respectively. The separated zones can be seen fluorometrically by ultraviolet irradiation or color reaction by dropping $SbCl_3$ reagent on the longitudinal section of the pushed-out column (Fig. 1).

Before 40 ml. of the developing solvent have passed through the column, the **I** and **II** zones come out of the column. The **III** zone is the vitamin A alcohol and its fluorescence or color reaction are the most intensive, being 3–3.5 cm. in length, beginning about 2 cm. below the upper surface of the column.

The color of the fluorescence, its intensity expressed as vitamin A alcohol, color of GDH reaction, its E value expressed as vitamin A are shown in Table I.

TABLE I

Fractionation of the Unsaponifiable Matter of Liver Oil Squalus sackleyi

Fraction		Color of fluorescene	Intensity of fluorescence as vitamin A		Color by GDH	E by GDH as vitamin A	
			μg./g.			*μg./g.*	
Non-A	**I**	reddish-green	3.0	24.8	light yellow	3.8	28.3
	II	greenish-orange	4.1		”	7.5	
	IV	greenish-blue	14.0		light violet	15.0	
	V	greenish-yellow	3.7		light yellow	2.0	
Total			164.0			164.0	
$\frac{\text{Non-A}}{\text{Total}}$			*per cent* 15			*per cent* 17	

1. *Stability of Vitamin A and Non-A Substances in* 10 *per cent Acetone in PB*—Vitamin A is in air relatively labile in 10 per cent acetone-PB but not so in CO_2. On the contrary, the non-A fractions remain unchanged in air within 120 minutes as shown in Table II. As it takes about 2 hours for the chromatographic separation by the indirect method the vitamin A alcohol can not be unchanged.

3. *The Absorption Curve of the Total USM and the Total Non-A substances in Liver Oil*—The absorption curves of total USM and the total non-A substances in liver oil were estimated and the curve corresponding to the actual vitamin A was obtained by the difference of both values. An example is shown in Fig. 3. It agreed practically well with the curve of vitamin A alcohol in benzene, the maximum being at 331

TABLE II

Stability of Vitamin A and Non-A Fractions in 10 per cent Acetone-PB

(Fraction I, II, IV and V are chromatographically separated from 150 mg. of liver oil *Theragra chalcogramma.* Vitamin A: 4.5 μg. The figures are E values (S55; 1 cm) determined by GDH method.)

Fraction	0 minute	90 minutes	120 minutes
Vitamin A (in air)	0.150	0.079	0.062
Vitamin A (in CO_2)	0.150	0.149	0.148
I (in air)	1.340	1.338	1.336
II (in air)	0.415	0.398	0.385
IV (in air)	1.388	1.392	1.389
V (in air)	0.689	0.688	0.686

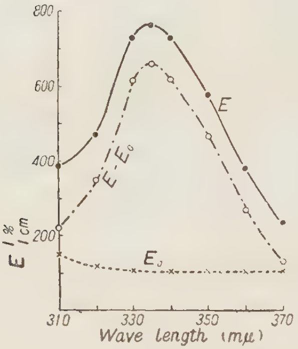

FIG. 3. Liver oil of *Reinhardtius matsuurae.*

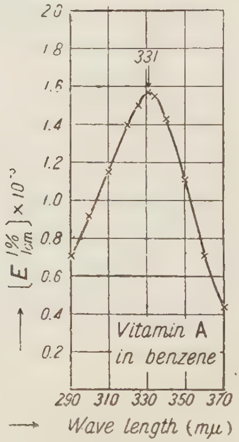

FIG. 4. Absorption curve of vitamin A in benzene.

mμ instead of 325 mμ as in ethyl alcohol. It was established by estimating pure vitamin A alcohol in benzene (Fig. 4).

4. *Ratios of Actual Vitamin A to the Total A Values of Liver Oils*—The actual vitamin A and the total A values of various liver oils are estimated with the results shown in Table III.

The percentage of the actual vitamin A to the total values are usually higher than 90 per cent. For liver oils poor in vitamin A the percentage of 50 per cent is observed. The actual values determined fluorometrically and colorimetrically agreed practically quite well.

TABLE III

Actual Vitamin A in Liver Oils

(F: Fluormometry. G: GDH. Figures are expressed as equivalent vitamin A)

Liver oil of		Total (a)	Non-A	Actual A (b)		b/a
		μg./g.	*μg./g.*	*μg./g.*	*I.U./g.*	*per cent*
Seriola quinqueradiate	F	4,205	200	4,005	12,000	95
	G	4,250	240	4,010	12,000	95
Squalus sackleyi	F	4,990	164	4,824	14,500	97
	G	4,990	165	4,725	14,200	95
Theragra chalcogramma	F	2,905	364	2,541	7,700	89
	G	2,880	225	2,655	8,000	92
Balaenoptera physalus	F	2,990	150	2,840	8,520	95
	G	3,340	440	2,900	8,700	87
Squalus sackleyi	F	296	146	150	450	51
	G	304	150	154	462	51
Vitamin A standard oil	F	3,300	10	3,290	9,870	100
	G	3,270	9	3,261	9,783	100
Scoliodon walbeehmi	F	2,202	246	1,956	5,870	89
	G	2,142	150	1,992	5,980	93
Katsuwonus vagans	F	1,815	372	1,443	4,330	80
	G	2,021	555	1,466	4,400	73
Balaenoptera borealis	F	14,760	980	13,780	41,300	93
	G	15,360	1,200	14,160	42,500	92

The proportion of non-vitamin A to vitamin A in various fish liver oils estimated by the extinction coefficient at 328 mμ, which have been reported in the literature is considerably higher in many oils of these types. It is possibly not because the oils which we used are atypical but the fluorometric and colorimetric determination method which are adopted by us are remarkably less influenced by non-A substances than extinction coefficient at 328 mμ as shown in experiments 1 and 7.

5. *The Absorption Curves of Vitamin A Alcohol Obtained by the Direct Method*—The absorption curves of the vitamin A fraction obtained by the direct method agreed quite well with that of pure vitamin A as are shown in Fig. 5–7.

6. *Separation of Kitol from Vitamin A*—Whale liver oil is saponified and theUSM is subjected to chromatography as described in the direct method. After washing twice with 2 ml. of PB (Anhydrovitamin A is removed completely), the column is rinsed twice with 2 ml. of benzene, whereby the filtrate contains the total vitamin A alcohol. By further

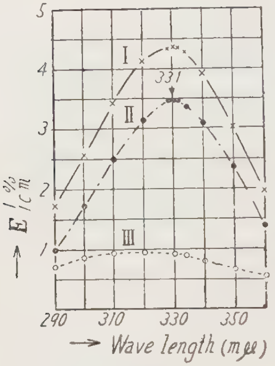

FIG. 5. Liver oil of *Katsuwonus vagans* in benzene.

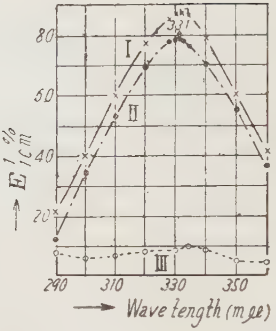

FIG. 6. Liver oil of *Rheinhardtius matsuurae* in benzene.

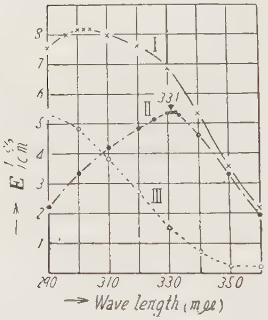

FIG. 7. Liver oil of *Balaenptera physalus* in benzene.

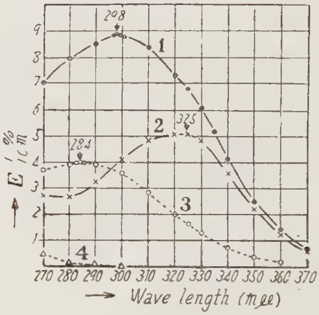

FIG. 8. Liver oil of *Balaenoptera physalus* in ethyl alcohol.

rinsing thrice with 2 ml. if 20 per cent acetone in PB, the total kitol is collected in the filtrate. The absorption curves obtained with each fraction and the total USM are estimated in alcoholic solution in order to be able to compare with the results in the literature (Fig. 8).

It is clear from the agreement of extinction curves and extinction maxima that the benzene eluate contains spectroscopically almost exclusively vitamin A alcohol alone and the acetone-PB eluate kitol alone.

7. *Properties of Kitol*—In order to study the properties of kitol, the USM of the molecular distillate residue of whale liver oil was subjected to chromatography on the weakly activated alumina and 19 mg. of kitol were obtained. Using kitol solution containg 7.6 μg./ml. alcohol

the extinction curve of kitol was estimated by Beckman spectrophotometer and $E^{1\%}_{1\text{cm}}$ 290 mμ was found to be 559 (Fig. 9) As the $E^{1\%}_{1\text{cm}}$ 290 mμ of crystalline kitol is according to Clough (*5*) 707, the purity of our preparation may be 79 per cent Using this solution the fluorescence intensity was estimated and found that the fluorescence of 600 μg. of pure kitol corresponds to 0.6 μg. of vitamin A alcohol, namely the fluorescence intensity of kitol is about 1/1000 of vitamin A alcohol on weight basis. Using the kitol solution we estimated colorimetrically by our GDH method and found that the pure kitol shows the E value corresponding to about 1/71 of vitamin A alcohol on weight basis. It follows that the GDH method is practically scarcely influenced by kitol. Further, we found that kitol gives the E value by Carr-Price reaction corresponding to 1/20 of A alcohol on weight basis.

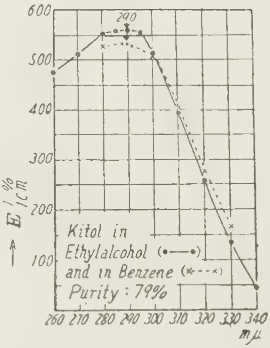

FIG. 9. Absorption curve of purified kitol.

8. *Comparison of the Direct Method with the Indirect Method*—Various liver oils are estimated at the same time by the direct and indirect methods. As shown in Table IV both values agree practically completely even in the solution containing much vitamin D as vitamin D standard oil.

TABLE IV

Comparison of the Actual Vitamin A Values by Direct and Indirect Methods

(The figures show the actual vitamin A contents)

Liver oil of	Indirect method (a)	Direct method (b)	a/b
	g./g.	*g./g.*	*per cent*
Katsuwonus vagans	1,550	1,570	99
Theragra chalcogramma	1,540	1,560	99
Squalus sackleyi	263	252	104
Vitamin D standard oil	260	258	101
Balaenoptera physalus	2,420	2,450	99

SUMMARY

Two methods of estimating actual vitamin A are described, one is the indirect and the other is the direct. The indirect method is the estimation of the actual vitamin A by substracting the non-A values from the total. The unsaponifiable matter of liver oil is usually composed of 4 fractions and can be detected by the fluorescence in ultraviolet light or color reaction with $SbCl_3$ reagent. The non-A fractions are stable but vitamin A is destroyed in the course of chromatography, which takes about two hours.

Using weakly activated alumina in form of adsorption column of short length the actual vitamin A fraction can be rapidly collected and estimated with complete recovery. The collected A fraction shows the absorption curve corresponding to the pure vitamin A alcohol, free from kitol. Vitamin A and kitol can be chromatographically sharply fractionated.

Kitol shows about 1/1000 of fluorescence of vitamin A alcohol and by GDH method about 1/71, by Carr-Price method about 1/20 of vitamin A on the weight basis.

The vitamin A values determined by the direct method agree quite well with those by the indirect method.

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The Journal of Biochemistry, Vol. 40, No. 2.

ON THE COMPONENTS OF BULL-FROG BILE (RANA CATESBIANA SHAW). IV

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Extensive studies on bile acids in various animals in this laboratory have led to the discovery of the stero-bile acids (*1*, *2*) and C_{27}- and C_{28}-steroids (*3*, *4*), which from their structural relationship seem to be intermediary metabolites of sterol to C_{24}-bile acids. Furthermore, it has been elucidated that all kinds of C_{24}-bile acids are formed by oxidation and then by reduction of cholic acid, successively. These experimental results suggest with good reason that many other degradation products of these stero-bile acids and steroids must be found in bile. Several compounds which might be in agreement with this assumption have already been found in bull frog bile: tetrahydroxycholane sulfate, $C_{24}H_{41}O_4NaSO_3$(m.p., 152°) and trihydroxy-bisnor-sterocholanic acid, $C_{26}H_{44}O_5$ (m.p., 172°) by Kurauti and Kazuno (*5*), and later α-trihydroxy-bisnor-sterocholanic acid (m.p., 172°) and β-trihydroxy-bisnor-sterocholanic acid (m.p., 190–5°) by Mabuti (*6*). These bile acids and steroids in bull frog bile are very interesting from a view-point of the metabolism of steroids. In this report the investigation of bull frog bile is described in more detail.

EXPERIMENTAL

Dried bile (2610 g.) collected from 2000 gall bladders of bull frog (*Rana catesbiana Shaw*) was finely powdered and extracted twice with boiling methanol for 2 hours. Methanol was evaporated on a steam-bath replacing last solvent with water. The aqueous solution was mixed with 10 per cent ferric chloride solution as far as the precipitate attained maximum, and was filtered after a while. Thus there were obtained two fractions: the precipitate (A) and filtrate (B).

The Precipitate (*A*)—The precipitate was dissolved in 5 per cent sodium carbonate and filtered. The filtrate was acidified and the formed precipitate was filtered, washed with water and dried. The dried material was treated with petroleum ether in a soxlet apparatus for removal of fatty substances, and then extracted with ether for 24 hours and ether-insoluble residue was then extracted with acetone for 24 hours.

α-Trihydroxy-bisnor -sterocholanic acid: The ether extract was evaporated to dryness, and the residue was dissolved in acetone. By concentrating the acetone solution, there were obtained needle-shaped crystals (m.p., 172°), showing no depression of the melting point mixed with the Mabuti's sample (*6*).

β-Trihydroxy-bisnor-sterocholanic acid: The acetone extract was evaporated to dryness and the residue was extracted with ether in a soxlet apparatus. The ether extract was again evaporated to dryness, the residue was dissolved in acetone, and the solution was concentrated. Needle-shaped crystals (m.p., 190–5°) were obtained.

Filtrate (*B*)—The filtrate (about 15 liters) was adjusted to weak alkaline with 2 per cent sodium carbonate, and filtered. The filtrate was concentrated on a steam bath to about one-third of its original volume. To the concentrated solution sodium chloride was added up to 3 per cent. After standing overnight, 30 g. of the precipitate (on dry basis) was separated by filtration. In the same manner, the other six fractions were obtained according to the final concentrations of sodium chloride as follows 5 per cent (35 g.), 8 per cent (110 g.), 12 per cent (42 g.), 20 per cent (15 g.), 30 per cent (12 g.), and more than 30 per cent (not estimated).

Tetrahydroxyhomocholane sulfate (m.p., about 161°): The above seven fractions were separately treated in a soxlet apparatus for removal of fatty substances with petroleum ether, and then extracted with ethyl acetate for 24 hours. By concentrating the ethyl acetate extract needle-shaped crystals (m.p., 152°) were yielded in all cases. The crystals separated by filtration were dissolved in alchol. This alcohol solution was treated with a small amount of water, and mixed with ether until it became tubrid. After standing overnight, there were obtained 4.9 g. of beautiful needle-shaped crystals (m.p., 161° with decomposition), showing positive Pettenkofer, but negative Hammarsten and digitonin-test, and pale yellow-brown Liebermann-reaction. This crystalline substance was very soluble in water and showed to be a saturated compound, since it neither reacted with bromine nor decolorized $KMnO_4$ solution, and it was found to contain sulfuric acid radical combined with metallic ion which could not be removed even by treatment with dilute hydrochloric acid; $[\alpha]_D^{31°}=+31.97°$ (0.1016 g. of the substance in 15 ml. of methanol).

Tetrahydroxyhomocholane sulfate (m.p., 188°): After removal of the sulfate compound (m.p., 161°), the fraction salted out with 3 per cent sodium chloride was extracted with alcohol for 24 hours in a soxlet apparatus. The alcohol extract, after the addition of a small amount of water, was mixed with ether until tubrbidity appeared. On standing there separated 2.5 g. of needle-shaped crytals (m.p., 188° with decomposition), showing the same physical and chemical properties as those of the sulfate compound (m.p., 161°); $[\alpha]_D^{31°}=+12.41°$ (0.1191 g. of the substance in 10 ml. of methanol).

Tetrahydroxyhomocholane sulfate (m.p., 199°): The similar extraction and treatment to the above ones, of the residue of every fraction salted out with 5–20 per cent sodium chloride yielded 7.3 g. of needle-shaped crystals (m.p., 199° with decomposition), having quite the same properties as the above described sulfate compound (m.p., 161°); $[\alpha]_D^{31°}=+15.01°$ (0.42 g. in 10 ml. methanol). After separation of this sulfate compound (m.p., 199°), the fraction salted out with 5 per cent sodium chloride yielded

0.7 g. of the sulfate compound (m.p., 188°).

Trihydroxyhomocholene (m.p., 177°): 2.5 g. of the sulfate compound (m.p., 188°) were dissolved in 100 ml. of 10 per cent alcoholic-NaOH solution and heated under reflux on a steam bath for 20 hours. To this solution water was added and alcohol was removed by evaporation. There occurred a precipitate, which was filtered after a while, washed with water and dried. The dried material was dissolved in methanol and the solution was treated with a small amount of water. On standing, there separated 1.9 g. of needle-shaped crystals (m.p., 177°), showing positive Pettenkofer-test. but negative Hammarsten- and digitonin-tests, and orange-yellow Liebermann-reaction. This substance reacted with bromine and decolorized $KMnO_4$ solution promptly.

Analysis Calcd. for $C_{25}H_{42}O_3 \cdot H_2O$, C 73.47%, H 10.85%
Found, C 73.91%, H 10.75%.

Trihydroxyhomocholene (m.p., 196°): By hydrolyzing 4.5 g. of the sulfate compound (m.p., 161°), 3.2 g. of trihydroxyhomocholene (m.p., 177°) were obtained. After separation of this substance, the mother liquor was concentrated and 0.2 g. of needle-shaped crystals (m.p., 196°) were obtained, which had the same properties as those of trihydroxyhomocholene (m.p., 177°).

Analysis Calcld. for $C_{25}H_{42}O_3 \cdot H_2O$, C 73.47%, H 10.85%
Found, C 73.35%, H 10.95%.
$[\alpha]_D^{30°} = +104.77°$ (0.019 g. in 15 ml. of methanol)

Trihydroxyhomocholene (m.p., 238°): The same treatment of 7 g. of the sulfate compound (m.p., 199°) yielded 5.1 g. of plate-shaped crystals (m.p., 238°), having the same properties as those of trihydroxyhomocholene (m.p., 177°).

Analysis Calcd. for $C_{25}H_{42}O_3 \cdot H_2O$, C 73.47%, H 10.85%
Found, C 73.83%, H 11.15%
$[\alpha]_D^{30°} = +57.99°$ (0.0255 g. in 10 ml. of methanol).

From the mother liquor, 0.4 g. of the above described trihydroxyhomocholene (m.p., 177°) were also obtained.

Trihydroxyhomocholane (m.p., 185–6°).: 0.2 g. of trihydroxyhomocholene (m.p., 177°) were dissolved in 5 ml. of glacial acetic acid, and catalytically hydrogenated by the aid of 0.1 g. of PtO_2, where 19 ml. of hydrogen were absorbed within 30 minutes, but no more in further 10 minutes. The solution was filtered, and to the filtrate a small amount of water was added. There separated 0.15 g. of long needleshaped crystals (m.p., 185–6°), which neither absorbed bromine nor decolorized $KMnO_4$ solution.

Analysis Calcd. for $C_{25}H_{44}O_3$, C 76.52%, H 11.30%
Found, C 75.86%, H 12.19%

Trihydroxyhomocholane (m.p., 199–201°): Catalytic hydrogenation of 0.2 g. of trihydroxyhomocholene (m.p., 238°) yielded 0.16 g. of plate-shaped crystals (m.p., 199–201°), which neither absorbed bromine nor decolorized $KMnO_4$ solution.

Analysis Calcd. for $C_{25}H_{44}O_3$, C 76.52%, H 11.30%
Found, C 76.47%, H 10.65%
$[\alpha]_D^{30°} = +4.14°$ (0.051 g. in 10 ml. of methanol)

DISCUSSION

Methanol extract of the dried bile of bull frog was fracionated with $FeCl_3$ into the precipitate of acidic substances and the filtrate of neutral substances. In the precipitate were found α-Trihydroxy-bisnor-sterocholanic acid (m.p., 172°) and β-trihydroxy-bisnor-sterocholanic acid (m.p., 190–5°). The structure of the former acid has been reported by Haslewood (*7*) to be 3,7,12-trihydroxy-coprostanic acid. The latter one was further subdivided into seven fractions which were salted out by adding sodium chloride at the concentration of 3, 5, 8, 12, 20, 30, and$>$30 per cent, respectively. After removal of fatty substances by treating repeatedly with petroleum ether, each fraction was extracted with ethyl acetate and three kinds of the sulfate compounds of tetrahydroxyhomocholane were obtained, of which the sulfate compound melting at 161° was obtained from all seven fractions (Table I). All of these three sulfate compounds were very hygroscopic, and had the same composition, containing sodium, which could not be removed by treatment with dilute hydrochloric acid. Upon hydrolysis these sulfate compounds, respective three kinds of tri-hydroxyhomocholene were yielded and from the filtrates sulfuric acid was detected. These trihydroxyhomocholenes were all unsaturated compounds, which absorbed bromine promptly and decolorized $KMnO_4$ solution. These results suggest that the starting materials of the sulfate compound have a tertiary hydroxyl group, linking with sulfuric acid, and one double bond. Since these trihydroxyhomocholenes gave negative digitonin-precipitation test, it is indicated that the hydroxyl group at C_3-position is α-configuration.

TABLE I

Properties of Tetrahydroxyhomocholane Sulfate, $C_{25}H_{43}O_4NaSO_3$, and Trihydroxy-homocholen, $C_{25}H_{42}O_3$

	Fraction	Melting point	$[\alpha]_D^{31°}$		Melting point	$[\alpha]_D^{15°(30°)}$
Sulfate ester	*per cent* 3—30	ca. 161°	+31.97	Homocholene	177° 196°	+ 34.36 +104.77
	3 and 5	188°	+12.41		177°	+ 34.36
	5 and 8∼20	ca. 199°	+15.01		177° 238°	+ 34.36 + 57.99

These trihydroxyhomocholenes were hydrogenated catalytically with

PtO_2 to corresponding saturated compounds, trihydroxyhomocholane, by equally taking up an equimolar amount of hydrogen (Table II).

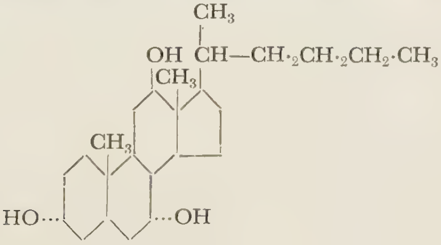
TABLE II

Propertion of Trihydroxyhomocholan

Homocholene	Melting point	Homocholene	Melting point	$[\alpha]_D^{12°(30°)}$
	177°		185°	+31.54
	238°		199—201°	+ 4.14
	196°			

Accordingly, three kinds of these trihydroxyhomocholenes are concluded to be probably isomers each other, being different either in the steric configurations of the C_7- and C_{12}-hydroxyl groups, or, more plausibly, in the position of the double bond. Two hydroxyhomocholenes out the three might also be isomers mutually, having a double bond at C_{15}-C_{16} or C_{14}-C_{15}, since different trihydroxyhomocholanes were obtained upon hydrogenation.

Recently, applying Kolbe's electrolytical technic to cholic acid, Kazuno and his co-workers (*8*) synthesized trihydroxyhomocholane (m.p., 186°), showing no depression of the melting point when mixed with a sample of trihydroxyhomocholane (m.p., 185–6°) obtained by hydrolyzing trihydroxyhomocholane sulfate (m.p., 188°), and hydrogenating trihydroxyhomocholene (m.p., 177°). From these facts, this trihydroxyhomocholane may be proved to have the following structure.



SUMMARY

1. From bull frog bile three kinds of tetrahydroxyhomocholane sulfate, melting at 161°, 188° and 199° with decomposition were ob-

tained. Upon hydrolysis these sulfate compounds yielded three kinds of trihydroxyhomocholene ($C_{25}H_{42}O_3$), melting at 177°, 196°, and 238°, respectively. The sulfuric acid radical was shown to be combined with a tertiary hydroxyl group of the starting sulfate compound in all cases.

2. Every trihydroxyhomocholene had one double bond, and α-configuration of the C_3-hydroxyl group.

3. Trihydroxyhomocholene melting at 177° and 238° were isomers, being different either in the steric configuration of hydroxyl groups at C_7- and C_{12}-positions or in the position of one double bond.

4. Trihydroxyhomocholane ($C_{25}H_{44}C_3$), melting at 185–6° and 199–201° were obtained by hydrogenation of trihydroxyhomocholenes, melting at 177° and 238°, respectively. The formar is $3(\alpha),7(\alpha),12(\alpha)$-trihydroxyhomocholane.

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THE SUCCESSIVE REDUCTION FROM NITRATE TO AMMONIA BY CELL-FREE BACTERIAL ENZYME SYSTEMS

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The reduction of nitrate is of general importance for higher plants, moulds and many species of bacteria. However, the studies on the actual agencies of this reduction process are very scanty* except for certain bacteria. Even in these cases, the studies are almost confined to the enzyme systems concerning to the first step of the reduction, namely from nitrate to nitrite. The present paper describes the successive reduction from nitrate to ammonia by a cell-free extract of a halotolerant bacterium.** ***

EXPERIMENTAL METHODS

Microorganism—A halotolerant bacterium having strong reducing activity for nitrate was isolated by Haruo Yamada. The microorganism was aerobic, motile and gram-positive rod bacillus and was identified by S. Hosoya aud M. Soeda as *Bacillus pumilus var.* In the following experiments, the organism grown for 48 hours on the peptone-broth-agar plate containing 3 per cent potassium nitrate and 10 per cent sodium chloride at pH 7.4,30° was used.

Enzyme Preparation—The bacteria cultured under these conditions were harvested and washed seral times with 10 per cent saline solution. Finally, the wased cells were heavily suspended in distilled water and completely dried by freeze-drying. The dried cells (0.3-1.0 g.) were suspended in 10 ml. of $M/10$ phosphate buffer (pH 6.8), and kept in an ice-box for 15–20 hours, and centrifuged. The suspernantant is slightly viscous, reddish and transparent liquid containing about 0.6–0.9 mg. N per ml. This supernatant containing nitrate reductase, nitrite reductase, and hydroxylamine reductase (abbreviated as NaR, NiR, and HdR, respectively) in cell-free state was used for the study of the reduction process from nitrate to ammonia. This was the first cell-free preparation of hydroxylamine reductase. Moreover it showed for stronger activity of NiR than that of Yamagata's (*4*). The dehydrogenase (*e.g.*, formic dehydrogenase) activities of this preparation were, however, weak. Therefore, each inorganic nitrogen compound was always reduced by coupling with the artificially added hydrogen donor system as will be shown later.

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* A. I. Virtanen, and N. Rautanen., in "*The Enzymes, Chemistry and Mechanism of Action.*" of J. B. Sumner and K. Myrbäck. Vol. II, Part 2. 1124–8 (1952).

** The results described in this paper were, for the most part, already presented at the 4th (1951, Tokyo) and the 5th (1952, Osaka) Symposia on Enzyme Chemistry (*1*, *2*) and the 2nd (1952, Paris) International Congress of Biochemistry (*3*).

*** In the addendum of the paper, *Australian J. Exptl. Biol. and Med. Sci.*, **24**, 167 (1946), it was reported that Back et al had obtained cell-free extract of E. coli which catalyse the reduction of nitrate, nitrite, hydroxylamine, fumarate, methylene blue and molecular oxygen by molecular hydrogen. But we could not know the details of their experiment.

Conditions of the Enzymatic Reactions—The reactions were always carried out anaerobically using Thunberg tubes at pH 6.8, 37° (pH value of 6.8 is optimum for each reductase). In order to observe the behaviour of each reductase independently, the reduction of each inorganic nitrogen compound was carried out by coupling with reduced form of methylene blue (abbreviated as Mb-H_2). This Mb-H_2 was prepared by reducing $M/100$ methylene blue (abbreviated as Mb) solution with hydrogen gas and Pd-$BaSO_4$ catalyst, and was anaerobically filtered and poured into Thunberg tubes.

Material and Methods of Estimation—

Preparation of hyponitrite (the method described by E. Diuero (*5*)): In measuring nitrate, a definite volume was taken from the reaction mixture, and poured into sulphuric acid solution and then the dye used as hydrogen donor was mixture was filtered. A definite volume of this colourless filtrate was used for estimation of nitrate. In the case of nitrite and hyponitrite, the saturated aqueous solution of oranyl acetate and kaolin or active charcoal were used for deproteinization and decoloration, and in the case of hydroxylamine and ammonia, trichloroacetic acid and kaolin were used.

Estimation of nitrate: The method described by A. C. Holler and R. V. Huch (*6*).

Estimation of Nitrite and Hydroxylamine—The method described by R. Novak and P. W. Wilson (*7*)*.

Estimation of Ammonia—The method of distillation at room temperature and nesslerization.

Detection of Hyponitrite—The method using potassium periodate and resorcinol described by A. S. Corbet (*10*).

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* It was sometimes considered that the methods of the same principle as that of the method for hydroxylamine mentioned above might estimate not only hydroxylamine, but also nitrohydroxylamic acid ($H_2N_2O_3$) (*8*, *9*). But it seems to be improbable that nitrohydroxylamic acid is produced as an intermediate in our experimental conditions.

RESULTS

The Reduction of Nitrate to Nitrite—The reduction of nitrate to nitrite, the first step of the successive reduction, has been investigated in our laboratory with enzyme preparation of *E. coli* to clarify the role of nitrate in biological oxidations and its reduction mechanism. The results obtained from these studies have been already reported (*11–16*). Cell-free extract from the halo-tolerant bacterium had very strong NaR activity. We obtained the same conclusion as obtained from the studies on NaR of *E. coli* in our laboratory, that nitrate was reduced according to the scheme proposed by Yamagata (*17*). This scheme on the mode of action is presented in Fig. 1. In order to study the reduction system apart from dehydrogenase system, we tried to carry out the nitrate reduction in an artificially simplified system, as shown in Fig. 2, with Mb-H_2 as hydrogen donor instead of formic acid-formic dehydrogenase system. We observed that nitrate was reduced also in this simplified system. This procedure and result are the same as in the case of *E. coli* investigated in our laboratory. By using this simplified system, the effect of several inhibitors on nitrate reduction was studied under the optimum condition. The results are shown in Table I. The action of NaR was strongly inhibited by cyanide and azide as NaR of *E. coli*. As this enzyme preparation had strong NiR activity, NaR activity was estimated by measuring the decrease of nitrate. Owing to the presence of NiR, it was very difficult to catch nitrite, the reduction

Fig. 1. The mode of action of the nitrate series reductases.

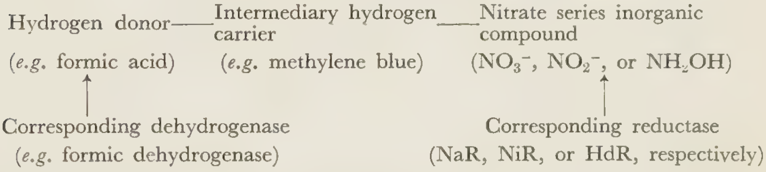

Hydrogen donor
(*e.g.* formic acid)
Intermediary hydrogen carrier
(*e.g.* methylene blue)
Nitrate series inorganic compound
(NO_3^-, NO_2^-, or NH_2OH)
Corresponding dehydrogenase
(*e.g.* formic dehydrogenase)
Corresponding reductase
(NaR, NiR, or HdR, respectively)

Fig. 2. Artificially simplified reductase system.

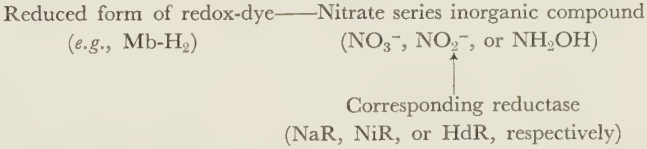

Reduced form of redox-dye
(*e.g.*, Mb-H_2)
Nitrate series inorganic compound
(NO_3^-, NO_2^-, or NH_2OH)
Corresponding reductase
(NaR, NiR, or HdR, respectively)

TABLE I

Effects of Inhibitors on Nitrate Reduction

Inhibitor	Final concentration of inhibitor	Inhibition		
		NaR	NiR	HdR
	M	*per cent*	*per cent*	*per cent*
KCN	10^{-1}		60	
,,	10^{-2}		40	
,,	10^{-3}	100	30	80
,,	10^{-4}	70		65
NaN_3	10^{-2}		20	
,,	10^{-3}	100	0	40
,,	10^{-4}	90		
NaF	10^{-1}	20	0	50

Reaction mixture:

In the case of nitrate reduction. 1.0 ml. enzyme preparation, 1.0 ml. *M*/100 KNO_3, 1.0 ml. *M*/2 phosphate buffer (pH. 6. 8), 4.5 ml. *M*/100 Mb-H_2, 0.5 ml. inhibitor of proper concentration; reaction time is 60 minutes.

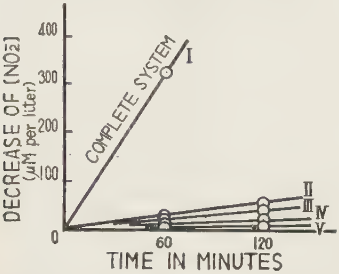
In the case of nitrite or hydroxylamine reduction. 1.0 ml. enzyme preparation, 0.5 ml. *M*/100 $NaNO_2$ or N_2H_2OH $\frac{1}{2}H_2SO_4$, 0.5 ml. *M*/2 phosphate buffer (pH. 6.8), 2.0 ml. *M*/100 Mb-H_2, 1.0 ml. inhibitor of proper concentration; reaction time is 180 minutes.

product, in a large amount. But by using a very active NaR preparation extracted from supersonics treated cells, we were able to catch nitrite amounting to 60–70 per cent of the decreased amount of nitrate.

The Reduction of Nitrite to Hydroxylamine—As described above, our enzyme preparation had strong NiR activity. Result obtained from the experiment on the mode of action of NiR is shown in Fig. 3. As in the case of NaR, the reduced amount of nitrite was remarkably decreased by omission of any one of the components from the complete system. From this fact, it can be considered that the mode of action of NiR is the same as that of NaR. Then in the case with Mb-H_2 as hydrogen donor, we were also able to observe nitrite reduction. The velocity was comparable to that of nitrite reduction with dehydrogenase system. And the reduction of nitrite only by Mb-H_2 never occurred. These results are presented in Fig. 4. In the research of the reduction products of nitrite, we found that hydroxylamine was one of the reduction products of nitrite. This result is presented in Fig. 5. The

Fig. 3. Nitrite redeuction according to the scheme:

$$\begin{tikzcd} \text{Formic acid} \arrow[r, dash] & \text{Mb} \arrow[r, dash] & NO_2^- \\ \text{Formic dehydrogenase} \arrow[u] & & \text{NiR} \arrow[u] \end{tikzcd}$$



Reaction mixture: 1.0 ml. $M/400$ $NaNO_2$, 0.5 ml. $M/2$ phosphate buffer (pH. 6.8). On addition to this, following components were arranged:

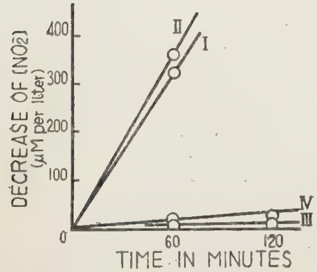
System	$M/10$ HCOONa	FD*	$M/100$ Mb	NiR preparation	Distilled water
	ml.	ml.	ml.	ml.	ml.
I	1	1	0.5	1	—
II	—	1	0.5	1	1
III	1	—	0.5	1	1
IV	1	1	—	1	0.5
V	1	1	0.5	—	1

* FD: formic dehydrogenase.

The suspension of dried cells of *Ps. fluorescens* (10 mg./ml.) which has scarcely any activity of reducing nitrate, nitrite and hydroxylamine was used as formic dehydrogenase preparation.

Fig. 4. Nitrite reduction by the simplified system:

$$\begin{tikzcd} \text{Mb-}H_2 \arrow[r, dash] & NO_2^- \\ & \text{NiR} \arrow[u] \end{tikzcd}$$

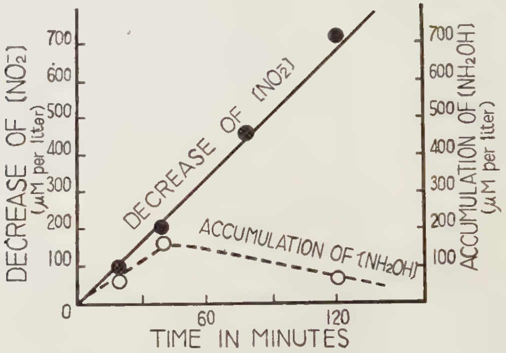


Reaction mixture: 1.0 ml. $M/400$ $NaNO_2$. On addition to this, following components were arranged:

System	$M/2$ (pH. 6.8) phosphate buffer	$M/10$ HCOONa	FD	$M/100$ Mb	$M/100$ Mb-H_2	NiR preparation	Distilled water
	ml.	ml.	ml.	ml.	ml.	ml.	ml.
I	0.5	1.0	1.0	0.5	—	1.0	—
II	0.5	—	—	—	2.0	1.0	0.5
III	1.0	—	—	—	2.0	—	1.0
IV	1.0	—	—	2.0	—	1.0	—

effect of several inhibitors on NiR activity was investigated with Mb-H_2 as hydrogen donor. As shown in Table I, NiR activity was not so strongly inhibited by cyanide and azide as NaR. NiR was completely inactivated by heat treatment at 100° for 10 minutes.

FIG. 5. The formation of nitrite reduction product.



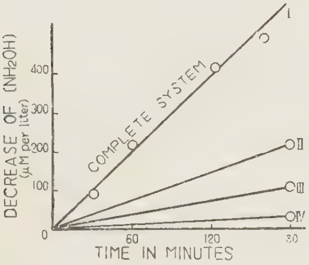
Reaction mixture: 1.0 ml. NiR preparation, 0.5 ml. $M/100$ $NaNO_2$, 0.5 ml. $M/2$ phosphate buffer (pH. 6.8), 2.0 ml. $M/100$ Mb-H_2, 1.0 ml. distilled water.

The Reduction of Hydroxylamine to Ammonia—As shown in Fig. 5, the amount of hydroxylamine accumulated by the reduction of nitrite decreased gradually with the time. This fact presented us the problem of hydroxylamine reduction. We expected the existence of an enzyme reducing hydroxylamine. Then, we investigated enzymatically this disappearance of hydroxylamine. As shown in Fig. 6, when any one of the components was omitted from the complete system, disappearing amount of hydroxylamine decreased remarkably compared with the complete system. From this result, the mode of action of HdR was ascertained to be the same as that of NaR or NiR. Then according to the same way as in the case of NaR or NiR, the reduction of hydroxylamine was carried out in the simplified Mb-H_2 system. The result is shown in Fig. 7. With the simplified Mb-H_2 system, the re-reduction product of hydroxylamine was investigated. One of the typical results is shown in Fig. 8. We could confirm that the reduction product was ammonia. Thus we have succeeded in demonstrating that the reduction of hydroxylamine to ammonia is catalyzed by a new enzyme, " Hydroxylamine Reductase." We also examined the effect of several inhibitors, the results of which are presented in Table I. HdR activity was inhibited by cyanide and azide. Heat treatment at 100° for 10 minutes inactivated HdR completely.

The Problem of Hyponitrite as an Intermediate—As shown in Fig. 9,

Fig. 6. Hydroxylamine reduction according to the scheme:

Formic acid——Mb——NH_2OH
↑ ↑
Formic dehydrogenase. HdR

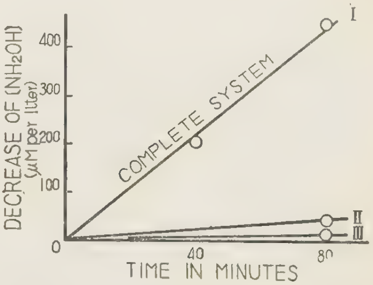


Reaction mixture: 1.0 ml. M/100 $NH_2OH \cdot \frac{1}{2}H_2SO_4$, 1.0 ml. M/15 phosphate buffer (pH. 6.8). On addition to this, following components were arranged.

System	HCOONa M/10	FD	M/100 Mb	HdR preparation	Distilled water
	ml.	ml.	ml.	ml.	ml.
I	1.0	0.5	0.5	1.0	—
II	1.0	0.5	—	1.0	0.5
III	1.0	—	—	1.0	1.0
IV	1.0	0.5	0.5	—	1.0

Fig. 7. Hydroxylamine reduction by the simplified system:

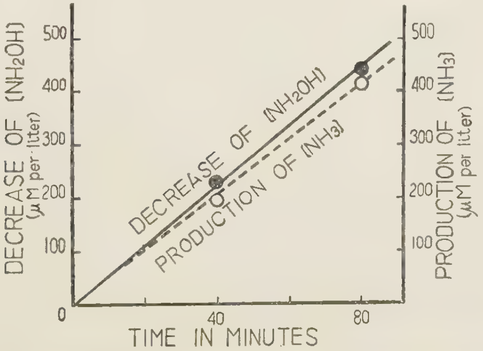
$Mb\text{-}H_2$——NH_2OH
↑
HdR



Reaction mixture: 0.5 ml. M/100 $NH_2OH \cdot \frac{1}{2}H_2SO_4$, 0.5 ml. M/2 phosphate buffer (pH. 6.8). On addition to this, following components were arranged.

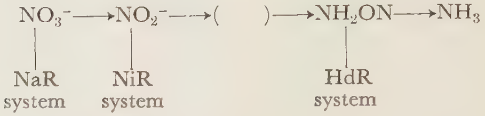
System	M/100 $Mb\text{-}H_2$	HdR preparation	Distilled water
	ml.	ml.	ml.
I	2.0	1.0	1.0
II	—	1.0	3.0
III	2.0	—	2.0

Fig. 8. The formation of hydroxylamine reduction product.



Reaction mixture: 1.0 ml. HdR preparation, 0.5 ml. M/100 $NH_2OH \cdot \frac{1}{2}H_2SO_4$, 0.5 ml. M/2 phosphate buffer (pH. 6.8), 2.0 ml. M/100 $Mb\text{-}H_2$, 1.0 ml. distilled water.

Fig. 9. The successive reduction process.



$$\begin{array}{ccccccc} NO_3^- & \longrightarrow & NO_2^- & \dashrightarrow & (\quad) & \longrightarrow NH_2ON \longrightarrow & NH_3 \\ | & & | & & & | & \\ \text{NaR system} & & \text{NiR system} & & & \text{HdR system} & \end{array}$$

two steps in the successive reduction process, namely the step from nitrate to nitrite and that from hydroxylamine to ammonia, are bivalent processes. While the step from nitrite to hydroxylamine is quadrivalent process. Considering such circumstances, the existence of hyponitrite $\left(\begin{array}{l} \text{N—OH} \\ \| \\ \text{HO—N} \end{array}\right)$ as an intermediate is presumed as is pointed out in the speculative scheme on nitrogen fixation process proposed by both schools of Wilson (*18*) and Virtanen (*19*). As other probable intermediates, dihydroxyammonia and nitramide, having the same molecular formula, $(HNO)_2$, should be considered. In the first place, we have tried to inspect the existence of hyponitrite by our bacterial extract with Mb-H_2 as hydrogen donor as usual. The accumulation of such compound in nitrification process was ever reported (*20*). But hyponitrite disappeared in the presence of small amount of Mb (it appears that our prepared Mb-H_2 practically contains such a small amount of Mb). Under such a condition, hyponitrite will be combined with Mb or catalytically decomposed by it. Other redox-dyes, nile blue, pyocyanine and brilliant cresyl blue did not cause such a disappearance of hyponitrite. So the reduction of hyponitrite was carried out with the reduced forms of nile blue, pyocyanine and brilliant cresyl blue. The experimental results are presented in Table II. As shown in this table, hyponitrite was not detected after 1 hour in the dye-free filtrate of deproteinized reaction mixtures (I, III, IV), but in the dye-free filtrate of not deproteinized reaction mixtures hyponitrite could be detected after 1 hour (I, III, IV). So, the remaining hyponitrite in the state of combination with proteins was qualitatively ascertained. Moreover, no colour change of these reduced dyes was observed in the system I, and the supposed reduction products of hyponitrite could not be recognized. This negative result for the possibility of the presence of hyponitrite as an intermediate was supported by the result of a rather indirect experiment presented in Fig. 10. The reduction from intrite to hydroxylamine which was catalyzed by the NiR system using reduced form of nile blue (abbreviated as Nb-H_2) instead of Mb-H_2 was not affected by the addition of hyponitrite. From these

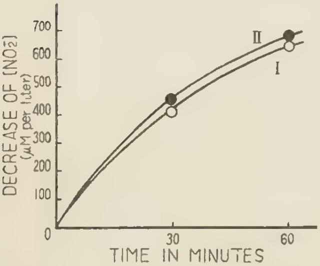
TABLE II

The Behaviour of Hyponitrite in the Simplified System with the Reduced Form of Nile Blue, Pyocyanine and Brilliant Cresyl Blue

System	Components of the system						Dectection of hyponitrite			
	$M/100$ $(NaNO)_2$	Enzyme preparation	20mg./ml. egg albumin aq.	$M/100$, dye of reduced form	$M/2$, phosphate buffer (pH. 6.8)	Distilled water	Dye-free filtrate of deproteinized reaction mixture		Dye-free filtrate of not deproteinized reaction mixture	
							Reaction time *minutes*			
							0	60	0	60
	ml.	*ml.*	*ml.*	*ml.*	*ml.*	*ml.*				
I	1.0	1.0	—	2.0	1.0	—	+	−	+	+
II	1.0	—	—	2.0	1.0	1.0	+	+	+	+
III	1.0	1.0	—	—	1.0	2.0	+	−	+	+
IV	1.0	—	1.0	—	1.0	2.0	+	−	+	+

Enzyme preparation and egg albumin by itself did not make the sign of Corbet test positive.

FIG. 10. The effect of hyponitrite on nitrite reduction in the simplified Nb-H_2 system.



Reaction mixture: 1.0 ml. enzyme preparation, 0.5 ml. $M/2$ phosphate buffer (pH. 6.8), 2.0 ml. $M/100$ Mb-H_2, 0.5 ml. distilled water. On addition to this,

(System I.): 0.5 ml. $M/100$ $NaNO_2$, 0.5 ml. distilled water.

(System II.): 0.5 ml. $M/100$ $NaNO_2$, 0.5 ml. $M/100$ $(NaNO)_2$.

experimental results, it may be concluded that hyponitrite cannot situate as an intermediate between nitrite and hydroxylamine. Further in the case of Mb-H_2, it seems to be probable that nitrite is reduced to hydroxylamine without passing hyponitrite as an intermediate, as in the case of Nb-H_2.

The Successive Reduction Process—As the bacterial extract contains the enzyme systems, NaR, NiR and HdR, it may be expected that the reduction of nitrate proceeds successively, according to the scheme shown in Fig. 9. In fact, we estimated the amounts of probable reduction products, nitrite, hydroxylamine, and ammonia, in the reduction of nitrate with Mb-H_2 as hydrogen donor. One of the typical data of the experimental results is shown in Table III. The total amount of the reduction products corresponds approximately to the decrease of nitrate. So, it may be concluded that the most part of nitrate is successively reduced to ammonia through the path as shown in Fig. 9.

TABLE III

Estimation of the Successive Reduction from Nitrate to Ammonia in the Simplified System

Reaction time	Decrease of $[NO_3^-]$	Accumulation of			Total of reduction droducts	Reecovery of reduction pronducts
		$[NO_2^-]$	$[NH_2OH]$	$[NH_3]$		
minutes	*μM/lit.*	*μM/lit.*	*μM/lit.*	*μM/lit.*	*μM/lit.*	*per cent*
0	0	0	0	(0)*	—	—
60	375	15	50	220	285	76
180	700	110	30	500	640	92

Reaction mixture: 1.0 ml. enzyme preparation, 1.0 ml. *M*/100 KNO_3, 1.5 ml. *M*/2 phosphate buffer (pH. 6.8), 4.5 ml. *M*/100 Mb-H_2. The final concentration of KNO_3 at time 0 in the reaction mixture is 1250 μM/lit.)

* This reaction mixture contained a small amount of endogeneous ammonia. So, the estimated values of ammonia were substracted by the amount of endogeneous ammonia, thus the values of produced ammonia were determined.

SUMMARY

1. Enzyme preparation having strong activity of reducing nirate, nitrite and hydroxylamine was extracted from a halo tolerant bacterium.

2. The existence of a new enzyme, " Hydroxylamine Reductase (HdR) " which catalysed the reduction of hydroxylamine to ammonia was confirmed.

3. Each of NaR, NiR and HdR catalysed the reduction of corresponding inorganic compound according to the scheme proposed by Yamagata.

4. The effects of inhibitors on NaR, NiR and HdR were studied with Mb-H_2 as hydrogen donor. The inhibitory effects of cyanide and azide suggest the contribution of some heavy metals to the enzyme systems.

5. It is probable that the most part of nitrate can be successively reduced to amonia through the path as shown in Fig. 9.

6. The existence of hyponitrite as an intermediate in this successive reduction process seems to be improbable.

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